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A SURVEY OF CHROMOSOME KNOBS IN MAIZE VARIETIES

MAHMOUD A. IBRAHIM

Head of the General Research Section, Ministry of Agriculture, Egypt, U. A. R.

Received May 11, 1959

CHROMOSOME knobs are enlarged pycnotic regions on the chromosomes. They stain deeply with acetocarmine and are best studied in the pollen mother cell in the pachytene stage.

McCLINTOCK (1930) was the first to report knobs on midprophase chromosomes of maize.

LONGLEY (1938) has demonstrated the presence of these knobs in maize and its related genera. perennial teosinte, annual teosinte and Tripsacum sp.

Chromosome knobs are now considered as structural characters which aid in the identification of many of the ten chromosomes of maize.

MATERIALS AND METHODS

About 150 open pollinated maize varieties were imported from all over the world. Seeds of perennial teosinte (*Euchlaena perennis* Hitchc.) were provided by DR. R. G. REEVES, at College Station, Texas (delivered by DR. M. F. EL-OBRASHY).

These varieties with six local maize varieties and a local annual teosinte (*Euchlaena mexicana* Schrad.), were planted on the farm of the Plant Breeding Section of the Ministry of Agriculture at Giza, Egypt, in the Nile, season of 1956.

Microsporocyte samples were collected from each variety, then killed and fixed in Farmer's fluid (glacial acetic acid, one part; 95 percent alcohol, three parts) for 24 hours. Preservation of the samples was made in 70 percent alcohol at a low temperature.

Belling's acetocarmine smear technique for staining the pollen mother cells was used in studying the chromosome knob number in the pachytene stage.

The enlargement was considered to be a knob if its size was within the measure used by LONGLEY (1941) beginning with the smallest, having a diameter of 0.33 μ and ending with the largest, having a diameter of 3.3μ. Enlargements less than the minimum limit were considered to be chromomeres, and those more than the maximum limit were taken as fused knobs.

EXPERIMENTAL RESULTS

Table 1 shows the varieties used in this study, with their sources, kernel type and size, kernel row number and chromosome knob numbers indicated.

Table 2 shows the geographical position of the sources of the varieties with the range of knob number in the varieties of each source.

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TABLE 1

The varieties, their sources, kernel type (D = dent, F = flint), kernel size (L = Large, M = medium, S = small), kernel row number and chromosome knob number

Variety	Source	Kernel			Chromosome knob number
		Type	Size	Row number	
White tuxpan	Arkansas U.S.A.	D	L	14–16	7
White synthetic	Arkansas, U.S.A.	D	M	14–16	3
Hickory king	Georgia, U.S.A.	D	M	8–10	6
Boone County white	Georgia, U.S.A.	D	L	14	3
Cassel	Georgia, U.S.A.	D	L	12–14	4
Alapaha corn	Illinois, U.S.A.	½D	M	12–14	1,2
Southians double	Illinois, U.S.A.	D	M	12–14	2,3
Limber cob	Illinois, U.S.A.	D	S	12	1,2
Garrick	Kentucky, U.S.A.	D	M	12–14	3,4
Gramling	Kentucky, U.S.A.	D	M	14	4
Weekleys prolific	Kentucky, U.S.A.	D	M	10	2,3
Tuxpan	Louisiana, U.S.A.	D	L	14	7
L.W. wadel greanweed Miss.	Louisiana, U.S.A.	D	L	14–16	6
Stiff stalk synthetic	Louisiana, U.S.A.	D	M	14–16	5
Johnson County white	Missouri, U.S.A.	D	L	14–16	6
Champion white pearl	Missouri, U.S.A.	D	L		2,3
Freed	Mississippi, U.S.A.	D	M	14–16	4
Blattel white	Mississippi, U.S.A.	D	L	14	3,4
Knighton little cob	Mississippi, U.S.A.	D	L	8–10	3,4
Long eared synthetic	Mississippi, U.S.A.	½D	M	10	3
Douthit prolific	Tennessee, U.S.A.	D	S	14–16	3
Ellis	Tennessee, U.S.A.	D	M	14–16	3,4
Highland	Tennessee, U.S.A.	D	M		6
Huffman	Tennessee, U.S.A.	D	L	12–14	5
Mexican June white	Tennessee, U.S.A.	D	L	12–14	8
Floridient white	Tennessee, U.S.A.	D	L	14–16	5
Pinkney	Tennessee, U.S.A.	½D	S	12–14	3
Biggs two ear	Texas, U.S.A.	D	L	12–14	3
Jellicorse	Virginia, U.S.A.	D	M	14	7
Highland horse tooth	Virginia, U.S.A.	D	S	12–14	2,3
Jones	Virginia, U.S.A.	D	M	14–16	3
Sure cropper white	Virginia, U.S.A.	D	L	12–14	6
Early king	Virginia, U.S.A.	D	L	8–10	2,3
Station wosly	Virginia, U.S.A.	D	M	12–14	3
Fred young	Virginia, U.S.A.	D	M	14	4
Gramling	Virginia, U.S.A.	D	M	14	4
Pride of Saline	Virginia, U.S.A.	½D	M	12–14	3
Thompson prolific	Virginia, U.S.A.	D	L	14–16	5
Wise prolific	Virginia, U.S.A.	D	L	14–16	7
American early	Middle Egypt, U.A.R.	D	L	14–16	5
Nab-El-Gamel	Middle Egypt, U.A.R.	D	L	8–10	4
Giza baladi	North Egypt, U.A.R.	F	S	12–14	2,3
Sabieni white	Faiyum Egypt, U.A.R.	F	S	10–12	2
Sabieni yellow	Faiyum Egypt, U.A.R.	F	S	10–12	0
Sawani white	Faiyum Egypt, U.A.R.	F	S	10–12	3
Maize K. T. 14	Baghdad, Iraq	F	S	12	4

TABLE 1—Continued

The varieties, their sources, kernel type (D = dent, F = flint), kernel size (L = Large, M = medium, S = small), kernel row number and chromosome knob number

Variety	Source	Kernel			Chromosome knob number
		Type	Size	Row number	
Yellow maize 35	Baghdad, Iraq	F	M	14	3
Yellow maize 27	Baghdad, Iraq	F	S	14	1
V.C. 31	Montpellier, France	F	L	12	1
V.C. 118	Montpellier, France	F	L	12	0
Burgenlandscher silo	Voldgsen, Germany	D	M	12	3
Gelber Silozahn	Voldgsen, Germany	D	M	12	3
Insubria 521	Rosarno, Italy	D	L	12	5
Bianco perla	Rosarno, Italy	F	S	12	5
Nostrano dell'Isola	Bergamo, Italy	F	S	12	2
P586	Boulaouane, Morocco	D	L	12	6
Dr. Dellile's Neue Kreuzgung	Lunteren, Netherlands	F	M	..	1
Lasspessmeyess silosalin	Lunteren, Netherlands	F	M	..	2
Muratha	Lunteren, Netherlands	F	L	10	2
V.C. 95	Elvas, Portugal	F	S	10	0
Hickory king	Pretoria, South Africa	D	L	8	4
Robyn	Pretoria, South Africa	F	S	12	2
Sahara	Pretoria, South Africa	F	M	10	1
American white flint	Pretoria, South Africa	F	S	12	1
Boesman	Pretoria, South Africa	F	S	12	0
Potchef stroos pearl	Pretoria, South Africa	D	L	10	3
Sennar shami E.C.R./580	Khartoum, Sudan	F	M	12	2
Akaba E.C.R./741	Khartoum, Sudan	D	S	12	3
Shand baladi E.C.R./492	Khartoum, Sudan	F	S	14	1
Hickory king E.C.R./492	Khartoum, Sudan	½D	S	8–10	2
Peruvian yellow E.C.R./320	Khartoum, Sudan	F	S	10	2
Silver king E.C.R./654	Khartoum, Sudan	D	M	..	3
Yellow maize	Beirut, Lebanon	F	M	12	1
Yellow boshar	Beirut, Lebanon	F	S	14–16	2
V.C. 10	Pontevedra, Spain	F	S	..	0
V.C. 11	Pontevedra, Spain	F	S	..	1
V.C. 26	Pontevedra, Spain	F	S	..	2
V.C. 50	Pontevedra, Spain	F	S	8	1
V.C. 59	Pontevedra, Spain	F	S	12	0
V.C. 62	Pontevedra, Spain	½D	L	10	1,2
V.C. 71	Pontevedra, Spain	F	M	8	0
V.C. 77	Pontevedra, Spain	F	M	..	2
Yellow dent O.P.	Samsun, Turkey	D	L	..	3
White dent O.P.	Samsun, Turkey	D	L	..	3
Yellow flint O.P.	Tarsus, Turkey	F	M	10	4
Red flint O.P.	Tarsus, Turkey	F	M	16	5

It will be noted, in Tables 1 and 2, that 39 American open pollinated varieties (imported from the southern states) were tested. All of these varieties were of the dent type. The knob number in them ranged from one to eight. The variety "Mexican June white," which is considered one of the oldest southern dents, has

TABLE 2

The geographical position of the sources of the varieties with the range of knob number in the varieties of each source

Sources of varieties	Latitude	Longitude	Altitude (meters above sea level)	Range of knob number
U. S. A.				
Arkansas	33° 33′ N	91° 11′ W	300	3–7
Georgia	33° .. N	84° 33′ W	180	4–6
Illinois	40° .. N	89° .. W	400	1–3
Kentucky	37° 32′ N	85° 20′ W	350	2–4
Louisiana	31° .. N	92° .. W	50	5–7
Mississippi	33° .. N	90° .. W	150	3–4
Missouri	38° 30′ N	29° 30′ W	500	2–6
Tennessee	36° .. N	86° .. W	450	3–8
Texas	32° .. N	100° .. W	300	3
Virginia	37° 30′ N	79° .. W	85	2–7
U. A. R.				
Middle Egypt	30° 2′ N	31° 13′ E	21	4–5
North Egypt	30° 7′ N	31° 7′ E	..	2,3
Faiyum, Egypt	29° 25′ N	30° 45′ E	..	0–3
Iraq				
Baghdad	33° 20′ N	44° 27′ E	25	1–4
France				
Montpellier	43° 36′ N	3° 55′ E	..	0,1
Germany				
Voldgsen	52° 6′ N	9° 36′ E	117	3
Italy				
Bergamo	45° 34′ N	9° 48′ E	146	2
Rosarno	38° 29′ N	16° 12′ E	750	5
Morocco				
Boulaouane	32° 49′ N	8° 6′ W	175	6
Netherlands				
Lunteren	52° .. N	5° .. E	12	1,2
Portugal				
Elvas	38° 53′ N	7° 9′ W	208	0
South Africa				
Pretoria	25° 45′ S	28° 4′ E	480	0–4
Spain				
Pontevedra	42° 34′ N	8° 43′ W	564	0–2
Sudan				
Khartoum	15° 35′ N	32° 35′ E	25	1–3
Lebanon				
Beirut	33° 53′ N	35° 30′ E	98	1,2
Turkey				
Samsun	41° 20′ N	36° 25′ E	1	3
Tarsus	36° 53′ N	30° 42′ E	40	4,5

eight knobs. The varieties imported from the other countries have a narrower range of knob number. In the varieties of these countries, except South Africa, the range did not exceed three knobs.

The wide range of knob number in the varieties of the U.S.A., South Africa and

the Egyptian Region may be explained on the basis that the weather in these countries is suitable for varieties, imported from different parts of the world, to be adapted.

Table 2 shows, also, that the varieties in the collection tested (which was imported from regions north to 40° latitude North) do not have more than three chromosome knobs.

The data obtained in the present investigation indicated that high knob number was highly associated with the large kernel size. The correlation coefficient between these two characters was $r = .696$.

There was an indication of positive association between high knob number and each of denting and high kernel row number. The correlation coefficient between high knob number and denting was $r = +.335$, and the five percent level of significance is $r = \pm .349$. The correlation coefficient between high knob number and high kernel row number was $r = .041$, and the five percent level is $r = \pm .232$.

DISCUSSION

Knob number was found to be constant for any individual plant, but it varies from strain to strain. Eighteen knob positions, including knobs on 15 out of the 20 arms of maize chromosomes, have been located.

McCLINTOCK (1934) found that, at a certain stage, nucleolar material is associated with all chromosomes, but later this material is drawn to a major organizing center or centers. Further study by LONGLEY (1939) has shown that knob-forming points exist even when no knob is formed, but no single point possesses a knob-forming power that overshadows all other points although there is a marked difference in the different points in their ability to form knobs.

LONGLEY (1938) found very few knobs on the chromosomes of most of the strains from the Northern Indian tribes. A slightly higher number was found in the southern varieties, and many knobs were observed in most of the varieties from New Mexico and Arizona. He reported 4.21 as the average knob number for North American maize strains. LONGLEY (1939) found also that less than one percent of the American varieties were without knobs, and in all of these varieties there was an appreciable piece of each chromosome adjacent to the fiber attachment that was knobless, and the short arms of chromosomes 5, 6, 7 and 8 seemed to be too short to reach a point at which knobs occurred frequently.

MANGELSDORF and CAMERON (1942) reported 7.9 and REEVES (1944) 9.25 as the average knob number for maize in Guatemala. MANGELSDORF and REEVES (1939) and REEVES (1944) suggested that maize might have developed as a highly domesticated plant in the Andean region and was spread from there to all parts of South, Central and North America. Its chromosomes were knobless, but during its migration northward it became hybridized with Tripsacum (a related genus with knobby chromosomes) in Central America, and from those hybrids new types of knobby chromosomes were derived.

BROWN (1949) in discussing this hypothesis pointed out that one would expect the North flints (which externally exhibit more Tripsacum influence than any

other group of varieties he studied) to possess many knobs. Actually they have few if any knobs. He concluded that more data are needed before one can safely regard knobs as a reliable indicator of Tripsacum germ plasm in Zea. He found also that the knob numbers in the Old Southern dents ranged from five to 12 and suggested that some of these varieties which have 11 or 12 knobs might have come directly from Mexico without having undergone much change in plant characters. The knob numbers in corn belt inbreds ranged from two to eight, and their over-all distribution was almost exactly intermediate between that of the Southern dents and Northern flints.

MANGELSDORF and REEVES (1959), in their further studies on the origin of corn, have found conflicting evidence to their hypothesis.

The data of the present investigation confirm the suggestion of LONGLEY (1938) that the number of chromosome knobs might give a clue to the geographical origin of the variety. No varieties having more than three knobs were found in the material tested in a region north to 40° longitude North.

This study shows also that when varieties migrated from one region to another, they were affected with the varieties located in the new region. The example for this is the variety Hickory king. BROWN (1949) found that the original type of this variety which might have come directly from Mexico to Georgia had 11 or 12 knobs. The writer found that the type which was imported from Pretoria, South Africa, had four knobs, and that which was imported from Khartoum, Sudan had only two knobs and had changed from dent type to half dent. The range of knob number in the other varieties imported from the same region in South Africa was zero to three and that in the varieties imported from Sudan was one to two knobs.

LONGLEY (1941) studied knob positions on teosinte chromosomes and found that all knobs on Guatemalan teosinte chromosomes are terminal. In Northern Guatemalan teosinte, 18 of the arms have been seen with a terminal knob, while in the teosinte from Southern Guatemala only 13 have been found terminated by a knob. He also found that the mean knob size for the northern group is 1.35 μ. while that of the southern group is 2.05 μ in diameter.

Perennial teosinte plants (*Euchlaena perennis* Hitchc.) which were tested in the present investigation had seven big knobs (more than 3.3 μ in diameter) and six ordinary knobs (between .33 and 3.3 μ). Eight of these 13 knobs were terminal. The local annual teosinte plants (*Euchlaena mexicana* Schrad.) had nine big knobs, six of which were terminal. The big knobs are considered fused ones.

The relationship between chromosome knob numbers and the agronomic characters has been studied by some investigators. MANGELSDORF (1947) suggested that knobs themselves may have no genetic effect, but that associated with them are segments of Tripsacum chromatin homologous or partially homologous to segments in maize. However, evidence of inheritance of such chromosome segments or blocks of genes which behave like single genes has been reported by him.

BROWN (1947) has found that the high knob number is correlated with high kernel row number, row irregularity, denting, absence of husk leaves, and many seminal roots.

VACHNANI (1950) has made a test for the interrelationship between knob

number and nearly all the agronomical and morphological characters of some inbred lines. No significant association was found, though some of the ear characters approached the five percent level of significance.

In the present study, high knob number is highly associated with large kernel size. There is also an indication of association between high knob number and denting and also high kernel row number.

SUMMARY

1. Chromosome knob numbers were determined in a collection of maize varieties from different parts of the world. Knob numbers ranged from zero to eight.

2. Varieties from regions north to 40° latitude North in the collection studied do not have more than three knobs.

3. The same variety names from different sources may have different knob numbers and different agronomic characters.

4. High knob number is highly associated with large kernel size and may be associated with denting and high kernel row number.

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EXTRANUCLEAR BARRIERS TO INTERSPECIFIC HYBRIDIZATION BETWEEN OENOTHERA HOOKERI AND OENOTHERA ARGILLICOLA

HARRY T. STINSON, JR.

Department of Genetics, The Connecticut Agricultural Experiment Station, New Haven, Connecticut

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THE extent to which heritable components outside the nucleus are involved in the various genetic events leading to species formation remains in question. The North American evening primroses of the subgenus *Euoenothera* offer an appropriate group of organisms to study some aspects of this problem. First, the cytogenetic behavior of Oenotheras provides a useful tool for the investigation of extranuclear inheritance, since it is relatively easy to obtain identical nuclei in combination with cytoplasmic elements from diverse origins. Exploiting this advantage, RENNER and his colleagues have focused special attention on the inheritance of a particular cytoplasmic inclusion—the chloroplast. From these studies has come convincing evidence that the chloroplast in Oenothera is an autonomous constituent of the genetic material, and further, that at least five genetically different plastid types exist in the strains of RENNER's collection (RENNER 1922, 1934, 1937; STUBBE 1959).

A second feature of the evening primroses that makes them suitable for analysis is the fact that the population structure and phylogenetic history of the North American Euoenotheras are known in considerable detail as a result of the studies of CLELAND and his students (see CLELAND 1956, 1958). There is thus available from each of the phylogenetic groups a representative sample of geographical races which have already been characterized cytogenetically.

Although some information has been collected on the nature of the plastid types present in the North American Euoenotheras (STINSON 1953), relatively little is known about the group as a whole, or about the bearing this information has on the phylogenetic history. Specifically, the question arises as to what part extranuclear elements, particularly the plastids, play in delimiting the various populations and species. One clue is perhaps provided by the phylogenetic studies mentioned above: it has been observed that the principal reason for failure to obtain viable hybrids between any two members of the North American population is chlorophyll deficiency in the F_1 offspring (see, for example, CLELAND 1950). This fact, coupled with the findings of RENNER's group on the inheritance of chloroplast characters in Oenothera, might suggest that intrinsic properties of the chloroplast contribute to the hybrid inviability. However, detailed genetic analyses are needed to ascertain the exact nature of the observed chlorophyll defects. The present paper reports such an analysis of interspecific crosses be-

tween *O. hookeri* and *O. argillicola*. An earlier, condensed report of certain of the results to be presented has been given elsewhere (STINSON 1958).

MATERIAL AND METHODS

Characteristics of the species: The two species *hookeri* and *argillicola* do not display, or do so in only minor degree, the cytogenetic aberrations characteristic of most members of the subgenus *Euoenothera*. Races of both species used in this investigation form seven pairs of chromosomes at meiosis, lack balanced lethals, and possess large, open pollinated flowers. The strains of the two species are further alike in having similar segmental arrangements, which are identical with or close to the arrangement taken to represent the putative ancestral chromosome condition for the North American Euoenotheras. In geographical distribution, however, the hookeris and argillicolas are markedly different; *hookeri* is found in California and Northern Mexico, with an eastward extension into Colorado, whereas *argillicola* occupies a range restricted to certain shale barrens in the Appalachian Mountains from Virginia to Pennsylvania. The two species are also clearly distinguished by their very dissimilar appearance; they differ in almost all characteristics except flower size.

The three *hookeri* races chiefly used were the California strains Johansen, Devils Gate and franciscana deVries. These strains are easily separated by several visible characters. Two of them—Johansen and Devils Gate—have the same segmental arrangement, which is the most common one yet encountered in the *hookeri* population. The segmental arrangement of franciscana deVries differs from the Johansen arrangement by a single interchange, and is the third most frequent *hookeri* arrangement. The three *hookeri* races are not only complex-homozogotes, but also approach genic homozygosity as a result of self-pollination from 16 to 18 generations.

The species *argillicola* was represented by the strains Douthat 1, Douthat 2, Douthat 3, Douthat 4a and Douthat 4b, all collected in Virginia. They are different in appearance, and are slightly different in their end arrangements. All five races had been maintained by self-pollination for five or six generations prior to their use in these studies, and none showed any signs of segregation, at least for morphological characters.

A third species, *O. parviflora*, served as a source of alien cytoplasm to which the genomes of *hookeri* and *argillicola* were transferred. *O. parviflora* is found in northeastern United States and eastern Canada, and unlike the two previous species is composed of typical, complex-heterozygotes, which are self-pollinating and breed true in spite of their heterozygosity. Such plants have all 14 chromosomes in one circle at meiosis, and produce only two kinds of gametes, each with a different set, or complex, of seven chromosomes. One kind of complex is transmitted chiefly through the egg (alpha complex), whereas the other complex is transferred mainly by the pollen (beta complex). In addition to their differences in end arrangement, the alpha and beta complexes of *parviflora* also give rise to quite different phenotypic effects. All beta *parviflora* complexes produce charac-

ters which resemble closely those found in *O. argillicola*. Two kinds of alpha *parviflora* complexes are recognizable, those which give *biennis*-like effects, and those which yield *strigosa*-like characters. Phylogenetically, the parvifloras appear to have arisen through hybridization between the progenitors of the argillicolas and ancient *biennis* and *strigosa* populations (see CLELAND 1958).

Two *parviflora* races, unlike in segmental end arrangement, phenotype, and geographical origin, served as sources of cytoplasm. One race, angustissima, was first described by GATES (1913) from a collection near Ithaca, New York. The alpha complex of angustissima shows strong *biennis* tendencies. The second race, Charlottesville, stems from a collection in the vicinity of Charlottesville, Virginia. The alpha complex of Charlottesville is identical segmentally to a complex of wide distribution in the Southeast—the so-called truncans complex (STEINER 1952). The truncans complex of Charlottesville, however, is perhaps more *biennis*-like in phenotypic expression than are most other truncans complexes. It should be pointed out that Charlottesville is unique among the *parviflora* races in possessing an alpha complex with the truncans arrangement.

Terminology and symbolism: The problem of terminology for extranuclear inheritance is often complicated by the fact that it is not always possible to associate the phenotypic character in question with a specific physical entity outside the nucleus. In the present investigation the initial transfers between genomes and cytoplasms presumably involved the *whole* cytoplasm, but the major interest is restricted to genetic systems that control chloroplast development. The question of whether the cytoplasmically inherited factors demonstrated to be part of these systems are located in the chloroplasts *per se*, or reside in other cytoplasmic elements is not only of theoretical interest, but is also pertinent to the practical problem of deciding on a terminology. This question will be considered later, but for the present it may be stated that available evidence appears sufficient to justify use of terms which refer to genetic properties of the chloroplasts themselves, as distinct from the remainder of the cytoplasm. Therefore, in dealing with the extranuclear components of inheritance the words plastid and plastome will be employed. The term plastome, as currently used by the German workers, designates the total genetic information localized in the chloroplast and does not have the same meaning as plastidome which refers to the total plastid complement of a cell in a morphological sense (see MICHAELIS 1957; SCHÖTZ 1958; STUBBE 1959).

For simplicity, the plastid types and genomes in the different species are abbreviated by upper and lower case letters respectively. Thus the genetic constitutions of major concern are symbolized as follows: *hookeri*, *H hh; argillicola*, *A aa; parviflora*, *P αpβp; hookeri* genomes associated with *parviflora* plastids, *P hh; argillicola* genomes combined with *parviflora* plastids, *P aa*.

Cultural methods: Seeds were germinated on moist filter paper in petri dishes, and with the first signs of germination the desired number of seedlings were planted in soil in the greenhouse. The seedlings not planted were kept in the petri dishes under artificial illumination, where the plants remained alive in a cotyledon stage of development for 2–3 weeks. Since many of the chlorophyll

abnormalities are evident in the cotyledons, the petri dish populations provided relatively large numbers of plants (usually 100–200) for observation. Thus, in several instances observations of chlorophyll phenotypes were made primarily on the petri dish populations.

RESULTS AND INTERPRETATION OF CROSSES

1. *Crosses between* hookeri (H hh) *and* argillicola (A aa): Interspecific crosses between *hookeri* and *argillicola*, made over a number of years between various races of the two species, have always given rise to chlorophyll deficient offspring, which at best have survived only to a young rosette stage of growth. This result was obtained irrespective of the direction in which the cross was made. The following strains of the two species have thus far been tested in one or more reciprocal interspecific crosses (progenies consisted of 100–200 plants): *hookeri* races—Devils Gate, franciscana deVries, franciscana Shull, hookeri deVries and Johansen; *argillicola* races—Douthat 1, Douthat 2, Douthat 3, Douthat 4a, Douthat 4b. The expression of the chlorophyll abnormality is not the same in the reciprocal hybrids. The cross *H hh* ♀ × *A aa* ♂ produces seedlings whose chlorophyll defect is readily apparent in the cotyledons of both petri dish and greenhouse plants. Depending upon the parental strains used, the color of the seedlings ranges from yellow to almost white. The hybrids have failed to survive beyond the cotyledon stage in every instance except for the cross *hookeri* deVries × Douthat 4b, where the offspring approach a pale green color.

The chlorophyll deficiency in hybrids from the reciprocal cross, *A aa* ♀ × *H hh* ♂, is less drastic and appears somewhat later in development; plants from this cross are consequently longer lived. The cotyledons of seedlings grown in petri dishes are usually normal green, while the cotyledons of greenhouse plants are, as a rule, pale green. Green plants transferred from the petri dishes to the greenhouse soon become pale green, suggesting that the color difference between petri dish and greenhouse plants is probably largely a matter of light intensity. In any event, with continued development the plants become progressively yellow-green. Generally, the yellowing process begins before the cotyledons are full grown, and the first true leaves are almost always yellow-green. Many plants die by the time the second and third pair of rosette leaves are formed. The surviving plants remain yellow-green to pale green, and some of them, although slow growing, may live for 2–3 months in the greenhouse. No plants have survived more than a few weeks in the field however.

Thus, the cross *A aa* × *H hh*, while producing less severe chlorophyll defects than the reciprocal cross, does, nevertheless, produce hybrids with chlorophyll defects that are lethal under ordinary conditions. It is possible that hybrids from the cross *A aa* × *H hh* could be grown to maturity if maintained under reduced light intensities. In this connection SCHÖTZ and REICHE (1957) have reported that certain chlorophyll deficient Oenothera hybrids which normally die in early growth stages may eventually become more or less green when grown under low light intensity for varying periods of time.

Clearly the chlorophyll abnormality present in hybrids from reciprocal crosses between *hookeri* and *argillicola* imposes a barrier to successful hybridization between the two species. In the sections that follow, the results of a successful attempt to overcome this barrier are presented.

2. *Transfer of* hookeri *and* argillicola *genomes to* parviflora *cytoplasm:* Underlying the operational procedure for the incorporation of a given genotype in a given cytoplasm is the assumption that the female parent contributes all the cytoplasm to the fertilized egg, or at least that any cytoplasmic contribution by the pollen parent is so small as to be discounted. It is well-known in Oenothera that this assumption is not always valid, since the pollen tube frequently transmits cytoplasmic elements in sufficient amounts to affect the phenotype and breeding behavior of the offspring. The problem is not always as serious as might be expected, however, especially for chlorophyll characters, since there are genetic means for detecting male plastids in the progeny. Thus, in the initial crosses aimed at the transfer of the genomes of *hookeri* and of *argillicola* to *parviflora* cytoplasm, it was assumed that cytoplasm was maternally inherited unless evidence to the contrary was found.

The procedure adopted for this transfer takes advantage of the segmental end arrangements of the particular races used, and of the absence of lethals in the two 7-paired species. Races of *parviflora* were crossed as female parents by the *hookeri* and *argillicola* strains to give a chromosome configuration of ⊙14 in each F_1 hybrid. As a consequence of regular alternate disjunction in the ⊙14 hybrids, the seven chromosomes of each complex separate as a unit at anaphase I. Since the *hookeri* and *argillicola* genomes are lethal free, 7-paired ^{h}*hookeri* · ^{h}*hookeri* (*hh*) and ^{h}*argillicola* · ^{h}*argillicola* (*aa*) segregates are recovered upon selfing the appropriate F_1 hybrids. The original female parent was *parviflora*, and, therefore, the 7-paired hookeris and argillicolas which appear in F_2 possess alien cytoplasm and plastids from *parviflora*. Taking *hookeri* as an example, the method is outlined in Diagram 1. Substitution of an *argillicola* male plant for *hookeri* in the diagram yields *aa* genome combinations in *parviflora* cytoplasm. Identification of the 7-paired segregates in F_2 was made on the basis of phenotype, and confirmed by cytological examinations. For convenience, the *hookeri* and *argillicola* races with *parviflora* cytoplasm will be referred to as the recovered races of the two species.

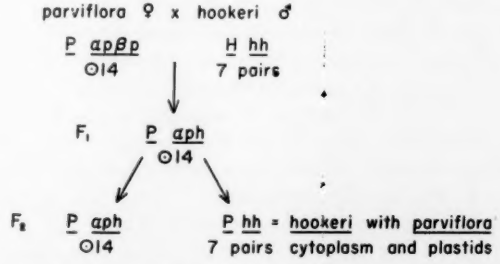

DIAGRAM 1.—Method of recovery of *hookeri* nucleus with *parviflora* cytoplasm and plastids.

To date the three *hookeri* races franciscana deV., Johansen, and Devils Gate have been recovered in the cytoplasm of the *parviflora* race Charlottesville. Only the latter two strains of *hookeri* have been adequately tested genetically, and consequently only their behavior will be considered. Three of the Douthat strains of *argillicola*—2, 3 and 4b—have likewise been recovered in Charlottesville cytoplasm, and Douthat 4b has in addition been isolated in the cytoplasm of the angustissima strain of *parviflora*.

In any F_2 population the recovered segregates are not absolutely uniform in phenotype, nor are they exactly identical to their original counterparts. With both *hookeri* and *argillicola*, however, the recovered strains are phenotypically very close to the original races, and there was never any doubt about the identification of the recovered types as to species and strain. This fact is even more striking when it is realized that most F_2 populations consisted of only 20 plants, and some of these were ⊙14 *aph* or *apa* individuals. It is quite clear that one is not dealing with the F_2 segregations that would be expected from hybrids between distinctly different species. In every case two phenotypic classes were immediately evident, and these corresponded to the two cytological classes, ⊙14 and seven pairs; closer inspection within each class revealed further segregations of a minor nature. The recovered plants selected for self-pollination and test crosses were judged most nearly to approach the original race in appearance.

The recovered strains of the two *hookeri* races, Devils Gate and Johansen, each differ from the original race in several minor respects, the most obvious and consistent differences being the slightly less speading habit and smaller buds and flowers of the recovered strains. The recovered Devils Gate strain also tends to show a transient lighter green color on the tips of young branches, and again at maturity to display a somewhat pale green leaf color. Both recovered *hookeri* races are fully fertile. The third *hookeri* race, franciscana deV., only recently recovered in *parviflora* cytoplasm, is also very similar phenotypically to its original counterpart. There is a difference in color evident in the early stages of growth, the recovered strain being somewhat paler green than the original race. At maturity this difference disappears.

Hybrid progenies between either the Charlottesville or angustissima strains of *parviflora* and the *argillicola* races always contain solid green and green, yellow-green variegated plants. Genetic analysis of the variegation indicates that both the green plants and green branches on variegated plants possess cytoplasm, or more precisely, plastids from the female *parviflora* parent (STINSON 1957, and unpublished). Therefore, only green plants or green branches were used for purposes of isolating the *argillicola* genomes in *parviflora* cytoplasm.

The three recovered *argillicola* races, Douthat 2, Douthat 3 and Douthat 4b, are very similar to the original races for all characters. As with the hookeris, some differences exist between the recovered and original strains, again most obvious in bud and flower size, and in growth habit. There has been no indication of chlorophyll differences between recovered and original strains. All three recovered argillicolas have exhibited normal fertility. In the case of Douthat 4b, several different lines have been established in each *parviflora* cytoplasm, and no two

lines are exactly alike. All, however, are unmistakably Douthat 4b-like, regardless of the source of *parviflora* cytoplasm.

3. *Breeding behavior of recovered races of* hookeri (P hh) *and* argillicola (P aa): (a) *P hh* ♀ × *A aa* ♂ —The results of this interspecific cross are markedly different from those obtained when the original hookeris are crossed by *argillicola*. As previously noted, crosses of the type *H hh* × *A aa* yield chlorophyll deficient seedlings which die while still in the cotyledon stage of growth. In contrast, the cross *P hh* × *A aa*, with either the recovered Devils Gate or Johansen as *P hh* parent, produces green, viable hybrids. The contrast in the two types of crosses is clearly seen in petri dish plants (Figure 1). The cross *P hh* × *A aa* does not, however, give exclusively green plants; with the formation of the first and second true leaves it becomes apparent that some plants are green, yellow-green variegated (Table 1). Discussion of these and subsequent variegated plants is deferred until later. Thus, in spite of their close phenotypic similarity, the recovered and orig-

TABLE 1

Crosses involving recovered hookeris and argillicolas

Cross	F_1			
	Total	Green	Variegated	Percent variegated
1) *P hh* females × *A aa* males				
Devils Gate × Douthat 1	60	49	11	18.3
Devils Gate × Douthat 3	110	105	5	4.5
Devils Gate × Douthat 4b	163	71	92	56.4
Johansen × Douthat 1	58	48	10	17.2
Johansen × Douthat 2	57	47	10	17.5
Johansen × Douthat 3	110	106	4	3.6
Johansen × Douthat 4a	30	18	12	40.0
Johansen × Douthat 4b	61	34	27	44.3
2) *P aa* females × *H hh* males				
Douthat 2 × Devils Gate	60	38	22	36.7
Douthat 2 × Johansen	59	15	44	74.6
Douthat 3 × Devils Gate	32	14	18	56.3
Douthat 4b* × Devils Gate	90	45	45	50.0
Douthat 4b* × franciscana deV.	124	85	39	31.5
Douthat 4b* × *hookeri* deV.	118	98	20	16.9
Douthat 4b* × Johansen	88	39	49	55.7
3) *P hh* females × *P aa* males				
Devils Gate × Douthat 2	75	75	0	0
Devils Gate × Douthat 4b*	289	289	0	0
Johansen × Douthat 2	111	111	0	0
Johansen × Douthat 4b*	179	179	0	0
4) *P aa* females × *P hh* males				
Douthat 2 × Devils Gate	59	59	0	0
Douthat 2 × Johansen	60	60	0	0
Douthat 4b* × Devils Gate	162	162	0	0
Douthat 4b* × Johansen	88	88	0	0

* Includes Douthat 4b strains with the two sources of *parviflora* plastids.

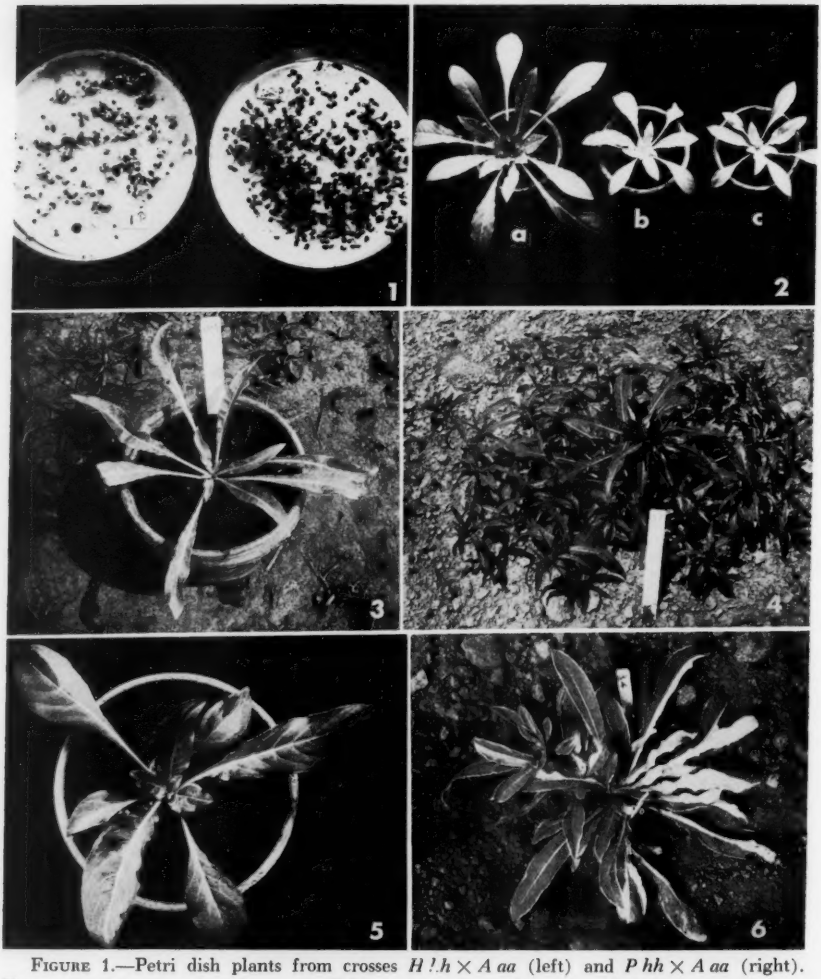

FIGURE 1.—Petri dish plants from crosses *H !h* × *A aa* (left) and *P hh* × *A aa* (right). FIGURE 2.—(a) green plant from cross *P hh* × *A aa;* (b) yellow-green plant from cross *A aa* × *P hh;* (c) yellow-green plant from cross *A aa* × *H hh.* FIGURE 3.—Yellow-green plant from cross *A aa* × *H hh.* FIGURE 4.—Green plant from cross *P aa* × *H hh.* FIGURE 5.—Variegated plant from cross *P hh* × *A aa.* FIGURE 6.—Variegated plant from cross *P aa* × *H hh.*

inal hookeris are revealed to be genetically different by their unlike maternal parent breeding behavior.

(b) *A aa* ♀ × *P hh* ♂ —This cross, the reciprocal of the preceding one, provides the first indication that extranuclear factors account for the genetic differences between the original and recovered hookeris. For when the interspecific cross is

made using *P hh* individuals as male parents, the offspring are not green but yellow-green. This result has been obtained in each of the reciprocals of the crosses reported in the preceding section. Moreover, every cross of the type *A aa* × *P hh* was always compared with the appropriate cross of the type *A aa* × *H hh*. The yellow-green plants from the two kinds of crosses are indistinguishable. It is evident, therefore, (1) that the *P hh* strains breed differently in reciprocal crosses with *argillicola*, green plants being produced only when the recovered strain acts as maternal parent (Figure 2a, b); and (2) that as male parents *P hh* strains behave as the original *H hh* races (Figure 2b, c).

(c) *P aa* ♀ × *H hh* ♂—The crosses of this type thus far investigated have had as female parents Douthat 2, Douthat 3, and Douthat 4b. The Douthat 4b strain, it will be recalled, has been recovered in two different sources of *parviflora* cytoplasm (Charlottesville and angustissima). The behavior of the recovered Douthat 4b strains in the two sources of *parviflora* cytoplasm has not been detectably different. Every cross of *P aa* × *H hh* has given progenies consisting of green and variegated plants, the latter with nongreen areas of yellow to yellow-white tissue (Table 1). Comparison between the cross *P aa* × *H hh*, which gives green (and variegated) plants, and the cross *A aa* × *H hh*, which yields chlorotic offspring, demonstrates that the recovered and original strains of *argillicola* do not breed alike as female parents. The difference in the maternal parent breeding behavior of original and recovered *argillicola* is illustrated in Figures 3 and 4 where two plants of the same age are compared. There is no doubt about the strikingly different outcome of the cross when a *P aa* plant replaces an *A aa* individual as female parent. (The chlorophyll deficient plant in Figure 3 died about two weeks after the photograph was taken.)

(d) *H hh* ♀ × *P aa* ♂—Each of the reciprocals of the crosses described in the preceding section has been grown. All have given rise to yellow or yellow-white offspring virtually identical to those produced by the comparable *H hh* × *A aa* cross. The recovered argillicolas, therefore, behave differently in reciprocal crosses with *hookeri*, and as pollen parents are indistinguishable from the original race. This genetic behavior exactly parallels that demonstrated for the recovered hookeris, and is compelling evidence for an extrachromosomal basis for the unlike breeding behavior of *P aa* and *A aa* plants.

4. *Additional test for plastome differences between original and recovered races:* Insofar as genetic effects on chloroplast function are concerned, the reciprocally different breeding behavior of the recovered strains strongly indicates that the original and recovered races differ solely in their plastomes, and not in nuclear constitution. It is recognized, nevertheless, that the recovered strains are almost certainly genically different from the original races, as suggested by phenotypic appearances. In fact, the method utilized for the transfer of *hookeri* and *argillicola* nuclei to *parviflora* cytoplasm means that the recovered strains might be expected to carry certain genes from the *α parviflora* complex as a result of crossing over in the F_1 hybrid stage of the recovery process. In the case of the hookeris an additional test has been possible which offers further evidence that the presence of *parviflora* genes does not determine the observed breed-

ing behavior of the recovered *hookeri* races in crosses with *argillicola*. The test consisted of a comparison of the maternal parent breeding behavior of *hh* individuals recovered in *parviflora* cytoplasm and *hh* individuals recovered in *hookeri* cytoplasm, the *h* genomes in each case having been associated with the same *α parviflora* complex in F_1.

The two kinds of recovered hookeris were obtained by essentially the same method described earlier. This time, however, the *hookeri* races Johansen and Devils Gate were crossed reciprocally with a plant of the Charlottesville strain of *parviflora*. Since the alpha complex of Charlottesville is transmitted by the pollen as well as the egg, the reciprocal F_1 combinations *H α ph* and *P α ph* are possible. The former F_1 plants yield on self-pollination *H hh* segregates (designated *H hh-rec*), while the latter F_1 combination produces *P hh* segregates. The four F_2 populations derived from the four reciprocal F_1's were grown in 1958. All *H hh-rec* and *P hh* segregates in the F_2 families were pollinated by the same *argillicola* race. Combining results from both *hookeri* strains, a total of 18 *H hh-rec* and 15 *P hh* plants were used in the testcrosses, and the progenies were grown in petri dishes in 1959. The *P hh* plants gave in every case green seedlings, whereas all *H hh-rec* plants gave yellow or yellow-white seedlings. These results are interpreted as follows: Provided that crossing over was similar in the reciprocal F_1 hybrids, and that the various crossover classes were equally viable in the two kinds of cytoplasm, the same crossover types should be represented among the *hookeri* genomes recovered in *hookeri* and in *parviflora* cytoplasm. Both *hookeri* recovered populations should be equally "contaminated" with *α parviflora* genes. If certain of these *parviflora* genes determined the outcome of crosses with *argillicola* males, one would expect to find plants among the two recovered types which breed alike in the testcrosses. In fact, however, all *P hh* segregates behaved differently from all *H hh-rec* segregates. Further, the behavior of *H hh-rec* plants was not demonstrably different from that of original *H hh* individuals. Even though the number of plants tested was not large, the fact that all *P hh* types behaved identically, as did all *H hh-rec* types, is difficult to reconcile with a strict genic interpretation. To explain the results on the basis of crossing over alone, it must be assumed that all 15 *P hh* plants acquired the necessary *parviflora* genes, while none of the 18 *H hh-rec* plants received the same *parviflora* genic contribution. This seems highly unlikely. A more reasonable conclusion is that the breeding behavior which *P hh* individuals exhibit as female parents in crosses with *argillicola* depends upon the presence of *parviflora* plastids, and not *parviflora* genes.

5. *Variegated plants from crosses involving recovered strains:* As previously mentioned, the two interspecific crosses employing the recovered strains of *hookeri* and *argillicola* as female parents—*P hh* × *A aa* and *P aa* × *H hh*—produce chlorophyll variegated plants as well as green plants (Table 1). In both crosses the variegation originally takes the form of irregular areas of green and nongreen tissue. With the development of subsequent rosette leaves the green and nongreen areas tend to become separated into discrete sectors, thereby giving rise to plants which show, usually, both a periclinal and sectorial chimeral ap-

pearance. Wholly green branches are almost always present on variegated plants, and rare solid colored nongreen branches are also found.

The variegated plants resulting from the two crosses are not identical, however, and it is this feature which distinguishes the two crosses. Variegation in plants from the cross *P hh* × *A aa* does not, as a rule, become evident until the first or second pair of rosette leaves are formed. The color of the nongreen areas is, from the beginning, yellow-green. In contrast, variegation in plants from the cross *P aa* × *H hh* is generally apparent in the cotyledons, and the nongreen areas are initially yellow-white. Thus, in the rosette stage, difference in color of the chlorophyll deficient tissue—yellow-green *versus* yellow-white—serves to differentiate the two types of variegated plants, as seen from comparison of Figures 5 and 6. This difference in the color of the nongreen tissues persists after the plants have broken rosette (as shown in Figure 6) but, interestingly, the yellow-white sectors eventually become yellow-green, and are indistinguishable from the yellow-green sectors on plants from the cross *P hh* × *A aa*.

Chlorophyll variegation is common in Oenothera interspecific hybrids, and has been explained by RENNER (first in 1922) on the basis of BAUR's hypothesis of biparental inheritance of chloroplast primordia (1909). According to this hypothesis the transfer of plastid primordia by the pollen tube results in zygotes with a mixture of maternal and parental plastids. The two types of plastids are sorted out during somatic cell divisions, thereby establishing cell lineages that contain exclusively one kind of plastid. If the two kinds of plastids react differently with the hybrid nucleus, variegated plants containing green and nongreen sectors arise.

Two lines of evidence may be cited to support the conclusion that the BAUR-RENNER hypothesis accounts for the variegation encountered in progenies from the two crosses *P hh* × *A aa* and *P aa* × *H hh*. First the color of the nongreen sectors (of rosettes) is in itself informative. If in the cross *P hh* × *A aa* the plastids in the yellow-green sectors are derived from the male (*A aa*) parent, the genetic constitution of these sectors is written *A ha*. This is the genetic constitution which also characterizes progeny from the cross *A aa* × *H hh*, described above. The latter cross, it will be recalled, produces yellow-green offspring, and their color is very similar to the yellow-green sectors on variegated plants from *P hh* × *A aa* crosses. Tissues of the type *A ha* in variegated plants and in ordinary hybrids, therefore, give rise to a yellow-green leaf color.

By a similar process of reasoning, the yellow-white sectors observed in variegated rosettes from the cross *P aa* × *H hh* are symbolized *H ha*. This genetic formula likewise describes offspring from the cross *H hh* × *A aa*, a cross that produces yellow-white hybrids. Somatic tissues with the constitution *H ha* are then identical phenotypically, irrespective of their origin.

This phenotypic similarity between chlorophyll deficient tissues in solid-colored hybrid plants and sectors on variegated plants applies not only to color, but also to the stage at which the deficiencies are first discernible. The chlorophyll defect in tissue of the genetic constitution *A ha*, both in hybrids from the cross *A aa* × *H hh* and in variegated plants from the cross *P hh* × *A aa*, is apparent

only after the first or second pair of rosette leaves arise, and normally is not seen in the cotyledons. Likewise, the chlorophyll deficiency in *H ha* tissues, in hybrids from the cross *H hh* × *A aa* and in variegated plants from the cross *P aa* × *H hh*, is first manifest at the same stage of development, in this case, the cotyledon stage. Thus, tissues presumed on the hypothesis to have the same plastid type and complex combination are seen to show a near exact correspondence in chlorophyll phenotype.

A second support of the BAUR-RENNER hypothesis is derived from crosses in which the male parents possess the same kind of plastid as the recovered female parents, that is, crosses in which both parents contain *parviflora* plastids. This condition is satisfied by the crosses *P hh* × *P aa* and *P aa* × *P hh*. No variegated plants appeared in any of the progenies from such crosses, as seen in Table 1 (crosses 3 and 4). On the hypothesis variegated plants are not expected, since both parents have *parviflora* plastids which develop normally with the *ha* hybrid nucleus. Male transmission of plastid elements would not, therefore, give rise to variegated hybrids, and would not in fact be detected by phenotypic appearances of the offspring.

It will be noted from Table 1 that the proportion of variegated plants varies rather widely in the different *P hh* × *A aa* and *P aa* × *H hh* crosses. These differences may well be partly the result of plastid competition, as described by SCHÖTZ (1954). The results suggest that not all *hookeri* or all *argillicola* plastids have the same relative reproductive potential, but more exact measures of the variegation, including estimates of the extent of green and nongreen areas, are needed to establish this point.

6. *Characteristics of the viable hybrids between* hookeri *and* argillicola: Both the green and variegated plants from the various crosses involving the recovered versions of the two species are normal in all respects. The hybrids, phenotypically intermediate between the parents, grow more vigorously than either parent, with the exception of rare variegated F_1 individuals in which considerable nongreen tissue is present. All hybrid plants grown to maturity have been fully fertile. Wherever they have been made, cytological examinations disclosed no difficulties in chromosome pairing, and the meiotic chromosome configurations expected on the basis of known segmental end arrangements were found in every case.

Several F_2 populations have been grown from crosses involving the recovered and original strains of Devils Gate and Douthat 4b (F_1 configuration = ⊙6, four pairs). When derived from green F_1 plants or green branches on variegated plants, the F_2 families display great variability for all plant characters, suggesting that segregation for a relatively large number of genes must be taking place. The range of variability is more restricted in F_2 populations derived from nongreen branches. The latter F_2 families always contain some chlorophyll deficient individuals which die before reaching maturity. The surviving plants show a decided leaning toward the phenotype of the parent which contributed the plastids to the F_1 nongreen branch. That is, when the self-pollination is made on a yellow-green branch of the constitution *H ha*, the F_2 progeny are definitely *hookeri*-like,

while the F_2 progeny from a yellow-green branch of the constitution *A ha* show a closer resemblance to *argillicola*. This difference in breeding behavior is expected; evidently, the F_2 plants which fail to survive because of chlorophyll defects are noncompatible combinations of *hookeri* genes and *argillicola* plastids (from *A ha* branches), or noncompatible combinations of *argillicola* genes and *hookeri* plastids (from *H ha* branches).

DISCUSSION

The starting point for the investigation of the role of extranuclear factors in interspecific crosses between *hookeri* and *argillicola* was the recovery of the genomes of each species in combination with an alien *parviflora* plastome. Conclusions drawn from the comparative breeding behavior of the original and recovered races are based on the assumption that the two kinds of races have identical, or near identical, nuclei but different plastomes. It is important, at the outset, to establish the validity of this assumption. First, with regard to nuclear composition, it is evident from phenotypic appearances that no recovered strain is absolutely identical to its original counterpart. These slight deviations in phenotype could be due to the presence of certain *parviflora* genes, acquired through crossing over in one, or both, genomes of the recovered races. It is also possible that the phenotype of the recovered races is altered by alien *parviflora* cytoplasm, since it must be assumed that the recovered races possess not only *parviflora* plastids, but, in fact, an entire cytoplasmic complement from *parviflora*. Reciprocal backcrosses between the original and recovered strains should provide the evidence needed to determine which of these alternatives is correct. Such backcrosses, now in their early stages, already suggest that genic differenecs are largely responsible for the slight phenotypic modifications observed in the recovered stocks. Although this preliminary evidence indicates that the recovered races possess certain *parviflora* genes, it is clear that these genes are not responsible for the different maternal parent breeding behavior of the recovered races. This view is supported by the reciprocally different breeding behavior of recovered races, and by the reciprocal recovery experiments. In terms of their effects on chloroplast development, therefore, the original and recovered races may be considered to have identical nuclei.

With respect to the extranuclear composition of the original and recovered strains, one fact that needs to be taken into account is the tendency of Oenothera pollen to transmit cytoplasmic elements (presumably plastid primordia) which affect the chloroplast content of the offspring. This behavior could influence the outcome of the recovery process since the assumption of exclusive maternal transmission of cytoplasmic constituents does not necessarily hold. In the cross *parviflora* × *argillicola*, pollen transmission of plastids does occur, for progenies from such crosses contain variegated plants. However, since analysis of the variegation shows the green branches to contain *parviflora* plastids, self-pollination of these branches insures that the recovered *argillicola* races possess *parviflora* plastids.

The situation with the recovered hookeris is somewhat more complicated in that male transmission of *hookeri* plastids would be difficult (with the races Devils

Gate and franciscana deVries) or impossible (with Johansen) to detect by inspection of F_1 families from the cross *parviflora* × *hookeri*. This is so because *hookeri* plastids develop normally, or nearly so, in combination with the F_1 *α ph* nucleus, and their presence would not be revealed by sectors of nongreen tissue. In other words, the F_1 plants would be genetic chimeras without an accompanying chimerical phenotype. It is evident, nevertheless, that male tranfer of *hookeri* plastids, if it occurred, did not interfere with the purpose of isolating *hookeri* nuclei in association with *parviflora* plastids. This is borne out by the fact that all recovered hookeris tested to date have behaved alike in testcrosses, and have differed from the original race in maternal parent breeding behavior. A recovered race which had original plastids would be expected to breed as an original *hookeri*, particularly in view of the evidence from the reciprocal recovery experiments. The conclusion appears justified, therefore, that the recovered strains of *hookeri* used in testcrosses did actually possess plastids from *parviflora*.

The recovery of 7-paired hookeris from *P α ph* F_1 plants is also interesting in light of STUBBE's findings (1959) of plastome effects on pollen viability. According to STUBBE, pollen grains which carry genomes of the *hookeri* or *strigosa* type are frequently nonfunctional when produced by plants with *parviflora* plastomes. Pollen inactivation obviously did not occur in the present case of the three different *hookeri* genomes combined with a *parviflora* plastome from the race Charlottesville; otherwise *hh* segregates would not have appeared in F_2.

In summary, the evidence supports the assumption that from the standpoint of effects on chloroplast development the original and recovered races possess identical nuclei but different plastomes.

Still to be considered, however, is the further assumption that the extranuclear component of the genetic system which controls chloroplast development is localized in the plastid and not in some other cytoplasmic element. Evidence pertinent to this point comes particularly from the extensive studies conducted by the German workers on chlorophyll variegation in F_1 Oenothera hybrids. Recently STUBBE (1957) and SCHÖTZ (1958) have advanced strong arguments for the contention, long held by RENNER, that the chloroplast itself possesses genetic properties. These two authors stress the point that individual cells which contain two morphologically distinct plastid types can regularly be observed in variegated hybrids, provided the microscopic examination is made on appropriate areas of a young plant. Moreover, transitional stages between the two plastid types are not seen. The morphologically distinguishable plastids are taken to represent the parental plastids, contributed by the egg and pollen tube, and which, because of their unlike genetic constitutions, respond differently to the same nucleus. The presence of single cells with distinct plastid types—the so-called mixed cells—is clearly called for by the BAUR-RENNER hypothesis of biparental, non-Mendelian chloroplast inheritance.

The existence of mixed cells in variegated plants is somewhat difficult to reconcile with interpretations that locate the genetic properties in nonplastid cytoplasmic elements. The demonstration of mixed cells thus offers strong evidence that the chloroplast *per se* possesses and transmits genetic information.

This view of the chloroplast has not gone unquestioned, however. On the basis of his electron microscope studies of chloroplast structure and development WETTSTEIN states (1957, 1959) that while there is uncertainty regarding the origin of chloroplasts, his studies indicate that "the complicated lamellate structure of the chloroplast can be built up from structural elements not notably different from those found in the ground cytoplasm." Thus, for higher plants, WETTSTEIN does not regard the concept of plastids as autonomous, self-duplicating bodies to be firmly established. Further, he believes it difficult to identify mixed cells with assurance, since cells with only one kind of plastid, either normal or defective, may show transitional stages of plastid development, and hence give the appearance of being mixed cells. Finally WETTSTEIN points out that a study (STUBBE and WETTSTEIN 1955) of certain mutant Oenothera plastids, inherited in a non-Mendelian fashion, demonstrated that the mutant plastids first reach a normal stage of development and then degenerate. This suggests to him that development of the plastid is not under plastome control. These criticisms of WETTSTEIN have been considered in detail by SCHÖTZ (1958), who does not believe them sufficiently compelling to abandon the idea of plastid-localized hereditary properties.

The problem of determining the location of the extranuclear components of the chloroplast system in Oenothera is perhaps still not finally resolved. However, genetic data from many investigations in Oenothera leave little doubt of the genetic continuity (as opposed to cytological continuity) of the extranuclear elements, which, together with the nucleus, determine plastid phenotypes. The ultimate decision as to the physical basis of these elements will certainly be determined in part by electron microscope studies of chloroplast origin, development, and multiplication. For the time being, the concept of a plastome, as previously defined, appears to be a useful and convenient working hypothesis.

The crosses involving the recovered and original races show without doubt that extranuclear factors play a critical part in determining the outcome of hybridization between *hookeri* and *argillicola*. A summary of the most important crosses is given in Table 2. Regardless of the plastome involved, all crosses between *hookeri* and *argillicola* produce the F_1 genome combination *ha*. The manner in which this nucleus expresses itself on chlorophyll formation depends on the kind of chloroplast present. With either *hookeri* or *argillicola* plastids the result is a lethal chlorophyll deficiency. When *parviflora* plastids are associated with *ha* nuclei, however, chlorophyll formation is normal. The chlorophyll abnormality in the *H ha* and *A ha* hybrids cannot, therefore, be solely attributed to properties of the nucleus. These observations lead to the view that the processes involved in chlorophyll formation are controlled by a genetic system which consists of nuclear and extranuclear components. The chlorophyll defect in reciprocal hybrids between *hookeri* and *argillicola* is explained as the consequence of a functional disharmony between the two components. The disruption of the chlorophyll apparatus thus sets up an effective barrier to gene exchange between the two species by causing death of hybrids at early stages of growth. Once the barrier is overcome by changing the plastome, the hybrid nucleus functions

TABLE 2

Summary of various interspecific crosses

Cross	Chlorophyll phenotype	Genetic constitution
hookeri females × *argillicola* males		
H hh × *A aa*	all yellow-white, inviable	*H ha*
P hh × *A aa*	1. green plants	*P ha*
	2. variegated plants	
	green sectors	*P ha*
	yellow-green sectors	*A ha*
P hh × *P aa*	all green	*P ha*
argillicola females × *hookeri* males		
A aa × *H hh*	all yellow-green, inviable	*A ha*
P aa × *H hh*	1. green plants	*P ha*
	2. variegated plants	
	green sectors	*P ha*
	yellow-white sectors	*H ha*
P aa × *P hh*	all green	*P ha*

in such a way that the vegetative and reproductive development of the species hybrids is fully normal.

The fact that *parviflora* plastids are compatible with the *ha* nucleus, whereas *hookeri* and *argillicola* plastids are not, must mean that *parviflora* plastids are inherently different from those in both *hookeri* and *argillicola*. But these differences in plastomes are not always apparent, even when the necessary genetic condition of identical nuclei is realized. This is illustrated in the present case by the recovery of *hookeri* and *argillicola* nuclei in association with the plastome of *parviflora*. The chlorophyll phenotypes of the recovered and original strains are, with the exceptions of the slight and transient differences in the *hookeri* races Devils Gate and franciscana deVries, identical. If observations were restricted to the recovered and original races alone, the conclusion could be drawn that *parviflora* plastids are virtually indistinguishable from those in *hookeri* and *argillicola*. The *ha* nucleus does, however, serve to differentiate the *parviflora* plastome from that of the other two species. Thus, as MICHAELIS (1954), among others, has emphasized, particular genomes are often needed to uncover cytoplasmic differences, and failure to recognize this undoubtedly allows hereditary variations in the cytoplasm to go undetected.

It is also possible to arrive at certain conclusions regarding the comparative properties of the chlorophyll-determining systems in *hookeri* and *argillicola*. Direct evidence bearing on the plastomes of the two species is furnished by the nature of the chlorophyll deficiency in the reciprocal hybrids *H ha* and *A ha*. Both hybrids show a chlorophyll defect, but as pointed out in the earlier description, with respect to intensity and time of onset the defect is manifest differently in *hookeri* and *argillicola* plastids (Table 2 and Figures 5 and 6). Thus, development of both *hookeri* and *argillicola* plastids is adversely affected by the *ha* genome combination, but the two types of plastids react in visibly different ways, and hence must be inherently different.

It is also readily apparent that the genic components which determine chlorophyll characters are unlike in *hookeri* and *argillicola*. This may be demonstrated by comparisons between the original species and the interspecific hybrids. Comparing *hookeri*, *H hh*, with the hybrid *H ha*, it is seen that replacement of an *h* by an *a* genome leads to defective *hookeri* plastids. Although this result does not necessarily mean that *a* genomes cause defects in *hookeri* plastids by direct action on the plastids, it does show that the *a* genome is different from the *h* genome in terms of genic effects on chlorophyll formation. Similarly, comparison of *argillicola*, *A aa*, with the hybrid *A ha* reveals that substitution of an *a* by an *h* genome results in abnormal *argillicola* plastids, again indicating that *hookeri* and *argillicola* genomes are different in their content of chlorophyll genes.

The differences which have been noted above in plastomes and genomes have also been found in the strains of RENNER's collection (STUBBE 1959).

Phylogenetic implications: Evidence from the studies on Oenothera phylogeny points to both similarities and differences between *hookeri* and *argillicola*. Both species have retained in common certain primitive characteristics which must have been present in the hypothetical ancestral *Euoenothera* population. These characteristics are: (1) mostly paired chromosomes; (2) alethal complexes; (3) open-pollinated flowers; (4) segmental end arrangements which are identical with or close to the putative ancestral arrangement. One difference between the hookeris and argillicolas is found in their unlike morphology. The lack of almost any phenotypic resemblance between the groups, as well as the wide range of variability observed in F_2 populations indicates that numerous genic differences exist between the two species. A second feature which distinguishes the two species is their geographical distribution, for they are currently separated by the width of the North American continent. Third, the hookeris and argillicolas are differentiated by their relationships with other phylogenetic groups. The closest relatives of the hookeris are the species *O. elata* and *O. strigosa*. The argillicolas, on the other hand, display little or no affinity with either the elatas or strigosas, and show instead a close connection with the parvifloras.

Taking into account both the similarities and differences between the hookeris and argillicolas, it has been possible to arrive at a reasonable conclusion concerning their positions in the phylogenetic scheme (STINSON 1953; CLELAND 1956, 1958). The argillicolas are regarded as relics of the first *Euoenothera* population to emerge, a population probably not far removed from the related subgenus *Raimmania*, and one which, in all likelihood, once occupied an extensive eastern range. In contrast, the hookeris have remained nearer the presumed center of distribution in Mexico or Central America. CLELAND (1958) has stated that the hookeris may be considered as either an ancient group, representing the original population, or alternatively as the most recent group to develop from the center of origin. No matter which of these alternatives is accepted, the conclusion is inescapable that the hookeris and argillicolas have been isolated geographically over a long evolutionary span. In summary, the common possession of primitive cytogenetic properties must mean that both groups ultimately trace their ancestry to a common origin; but the facts from phenotypic appearance, geographi-

cal distribution and species relationships argue that the two populations diverged early in their evolutionary history. Their common ancestry is thus a remote one.

How may the findings of the present study be applied to this picture of a remote common ancestry? First, it is clear that because of the nature of the genetic systems controlling chloroplast function in the two species, they are not only geographically, but also reproductively isolated. The analysis of these systems led to the conclusion that both the nuclear and extranuclear components are so different in the two species that the plastids of one species cannot function properly with even a single genome of the opposite species. Considering the conclusions drawn above relative to the phylogenetic histories of *hookeri* and *argillicola*, it is probable that this divergence in genomes and plastomes arose during the period of spatial isolation where the two populations were exposed to different environments and selective pressures. The differences between *hookeri* and *argillicola* revealed by the comparative genetics of chloroplast development are thus additional aspects of the total genetic divergence between the two species. But the differences in genomes and plastomes are of special biological interest because they alone, among the genetic properties of the two species, constitute the mechanism responsible for hybrid inviability.

In this connection it should be emphasized that the barrier to interspecific hybridization imposed by the interaction of genome and plastome operates in reciprocal crosses. STEBBINS (1958) has commented that gene cytoplasmic interactions are perhaps not likely to be effective genetic isolating mechanisms, since crosses which fail in one direction can frequently be made in the opposite direction, and hence gene exchange is not precluded. This situation does not hold in the present case; the barrier is complete, and the final criterion for species delimination—reproductive isolation—is realized.

If the results of this study, based on analysis of a relatively few strains of each species, are taken to be representative of interspecific hybridization between *hookeri* and *argillicola*, it may be concluded that in Oenothera at least cytoplasmic factors can play a part in speciation by their involvement in genetic systems which act as isolating mechanisms. It is interesting to note that CASPARI (1948), after reviewing cytoplasmic inheritance in many organisms, came to the conclusion that the significance of cytoplasm in evolution would most likely be found in its ability to act as a barrier to interspecific hybridization. JONES (1951), who noted that the fundamental properties of pollen fertility and chlorophyll development are often partly determined by cytoplasmic elements, came to the same conclusion.

SUMMARY

Reciprocal crosses between the two 7-paired lethal-free species, *O. hookeri* and *O. argillicola*, produce chlorophyll deficient offspring which die in the early seedling or rosette stage. These chlorophyll defects were demonstrated to arise from an incompatibility between the chloroplasts of either species and the hybrid nucleus. Proof of this contention was the discovery of a plastid type in which the

hybrid nucleus failed to produce the chlorophyll disturbance. This was accomplished by recovery of the nuclei of three races of both *hookeri* and *argillicola* in association with plastids derived from a third species, *O. parviflora*. The recovered races are green and fertile. When made using either kind of recovered race as female parent, the interspecific crosses gave green and variegated offspring, both classes being fully viable. Evidence is cited which supports the view that the variegation results from the transmission of plastids through the pollen. Crosses in which both parents were recovered strains yielded only green plants. The behavior of the recovered races in reciprocal crosses, and testcrosses involving reciprocally recovered *hookeri* stocks showed that the difference in maternal parent breeding behavior of original and recovered races was not due to contamination of the latter races with *parviflora* genes, but instead depended upon the presence of *parviflora* plastids in the recovered strains. It has been possible, therefore, by genetic manipulation of extranuclear elements to overcome the barrier to interspecific hybridization between *hookeri* and *argillicola*. The F_1 hybrids thus obtained are vegetatively vigorous and reproductively normal, as are the F_2 populations which have been grown.

Reasons are given for the tentative assumption that the extrachromosomally inherited component of the genetic system controlling chloroplast function is the chloroplast itself rather than some other cytoplasmic element. On this basis, it was pointed out that the plastids of *hookeri* and *argillicola* are not only different from those in *parviflora*, but also from each other. In terms of their effects on chloroplast development, the genomes of *hookeri* and *argillicola* are likewise dissimilar. These plastid and genome differences, which account for the reproductive isolation between *hookeri* and *argillicola*, are taken to support the conclusion that, in spite of their similar cytogenetic properties, the two species are only remotely related.

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HIGH NEGATIVE INTERFERENCE AT THE AM LOCUS IN NEUROSPORA CRASSA[1]

J. A. PATEMAN[2]

Botany School, University of Melbourne, Parkville, Victoria, Australia

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A NUMBER of independently induced alleles (PATEMAN and FINCHAM 1958) are known at the "amination deficient," *am*, locus in *Neurospora crassa*. In crosses between strains carrying different alleles, wild type ascospores were found in low frequency amongst the progeny. In an attempt to elucidate the mechanism of this wild type formation, marker genes on either side of the *am* locus were used in crosses between strains carrying the two alleles which gave the highest frequency of wild types among their progeny (PATEMAN and FINCHAM 1958). These alleles were previously referred to as am^{47} and am^{29} (PATEMAN 1957, 1958) where their origin is described, but are now designated am^2 and am^3, respectively. The first series of marked $am^2 \times am^3$ crosses gave unexpected results which were briefly described by PATEMAN (1958). This present paper describes the results of a further series of marked crosses and the genetic analysis of a number of the wild type progeny.

MATERIALS AND METHODS

The mutant strains used for markers were 37401 (inositol-requiring), *inos*, and a morphological mutant known as "spray", *sp*. The mutant *sp* was kindly supplied by DR. R. W. BARRATT who also first located the *am* locus between *sp* and *inos* on the right arm of chromosome V. The order of markers with reference to the centromere is *sp-am-inos*. Random ascospore platings of the appropriate crosses (PATEMAN, unpublished) gave map distances of 5–8 cM for the *sp–am* region and 2–3 cM for the *am–inos* region.

All vegetative cultures were kept on Fries No. 3 medium supplemented with 10 μgm per ml of inositol where necessary. All crosses between strains were made on a medium favouring sexual reproduction (WESTERGAARD and MITCHELL 1947) supplemented with 10 μgm per ml inositol.

EXPERIMENTAL RESULTS

First series of marked $am^2 \times am^3$ *crosses:* In the first series of experiments, briefly described by PATEMAN (1958), two types of cross were set up, *sp* am^2 $inos^+ \times sp^+$ am^3 *inos* and *sp* am^3 $inos^+ \times sp^+$ am^2 *inos*. Ascospores from these

[1] This work was supported in part by a grant from the Anti-Cancer Council of Victoria, Australia.

[2] Present address: Department of Genetics, University of Cambridge, England.

crosses were spread on Fries No. 3 medium plus 1.5 percent agar, 1–5 percent sorbose, 0.75 percent sucrose, 10 μgm per ml inositol and 0.02 M glycine contained in Petri dishes. The glycine strongly inhibits the growth of *am* ascospores with relatively little effect on *am*$^+$ spores. The sorbose induces a colonial growth habit in any *am*$^+$which develop and facilitates their isolation. The plates were kept at 60°C for 35 minutes immediately after spreading and then incubated at 25°C. The total number of live ascospores (after two days' incubation) was estimated by counting the germinated ascospores in 20 sample fields of each plate, with a binocular dissecting microscope. The plates were searched after two days' incubation; the majority of the *am*$^+$ ascospores could be recognized at this stage by their vigorous growth, and they were isolated. Any *am*$^+$ which were slow in developing, or had been missed in the first search, were picked up as barely visible colonies after a further one to two days' incubation, and were isolated. The isolated *am*$^+$ were then scored for spray and inositol requirement. In the case of those isolates where it was not certain that only the single *am*$^+$ ascospore had been transferred, the *am*$^+$ was crossed to an *am* strain, and several single ascospore *am*$^+$ progeny from this cross were also scored for spray and inositol requirement. This procedure ensured that the marker alleles associated with the *am*$^+$ allele were accurately known in every case. The results obtained from these *am* × *am* crosses are given in lines 5 and 6 of Table 1, together with the results (lines 1, 2 and 4) from crosses between strains carrying the same allele at the *am* locus. The latter crosses taken from the results given by PATEMAN and FINCHAM (1958) are included to demonstrate that the *am*$^+$ obtained from crosses between different *am* alleles are not due to back mutation or contamination.

The distribution of the marker alleles in the *am*$^+$ strains from these *am* × *am* crosses is remarkable, and does not correspond to that expected on the hypothesis that the *am*$^+$ are produced by classical crossing over. There is a great excess of strains which are parental in type with respect to the markers *sp* and *inos*, and, consequently, would have to be regarded as double crossovers on the classical model. In the two types of cross, which are complementary with respect to the outside markers, the frequencies of the two crossover types and of the two parental types should be reversed, but this is not so. Furthermore, whatever the assumed linear order of the two alleles within the *am* locus, one of the two types of cross should give the genotype *sp*$^+$ *am*$^+$ *inos*$^+$ as the most frequent kind of recombinant. This genotype would result from a crossover between the two *am* alleles with no other accompanying crossover between the markers *sp* and *inos*. In fact, no strain with the genotype *sp*$^+$ *am*$^+$ *inos*$^+$ was found in the sample of 47 wild types. Finally, although both the markers *sp* and *inos* would be expected to show a 1:1 segregation over-all, there is a significant excess of *sp* in both types of cross.

Second series of marked am^2 × am^3 *crosses*: It seemed possible from the previous results that a segment of chromosomal material carrying the *am*^{2} allele might be preferentially included during the formation of a wild type at the *am* locus. In order to check this point, crosses were set up between strains carrying *am*^{2} and

TABLE 1

Frequency of am$^+$ *from* am × am *crosses and the distribution of the marker alleles at the spray and inositol loci*

Cross	Genotypes				Medium	No. of *am*$^+$	No. of live ascospores in thousands	Frequency per 10^6 live ascospores
	sp$^+$ *inos*$^+$	*sp inos*	*sp*$^+$ *inos*	*sp inos*$^+$				
am^2 × *am*^{2}	..	..	..	..	Sorbose	0	465	....
am^3 × *am*^{3}	..	..	..	..	Sorbose	0	585	....
am^2 × *am*^{3}	..	..	..	..	Sorbose	14	651	21.54
sp am^2 *inos*$^+$ × *sp*$^+$ *am*^{2} *inos*	0	0	0	0	Sorbose	0	209	....
sp am^2 *inos*$^+$ × *sp*$^+$ *am*^{3} *inos*	0	6	0	17	Sorbose	23	1,549	14.85
sp$^+$ *am*^{2} *inos* × *sp am*^{3} *inos*$^+$	0	8	7	9	Sorbose	24	1,214	19.77
sp am^2 *inos* ×	4	1	0	2	Sorbose	7	285	24.57
sp$^+$ *am*^{3} *inos*$^+$	2	8	6	3	No sorbose	19	596	31.88
sp$^+$ *am*^{2} *inos*$^+$ ×	4	2	1	4	Sorbose	11	624	17.63
sp am^3 *inos*	3	2	1	0	No sorbose	6	524	11.45
Total	..	..	..	..	....	104	5,443	19.11

am^3 and the outside markers in the other two possible combinations, i.e., *sp*$^+$ *am*^{2} *inos*$^+$ × *sp am*^{3} *inos* and *sp am*^{2} *inos* × *sp*$^+$ *am*^{3} *inos*$^+$. The results from these crosses should show if there was always an excess of those marker alleles put into the cross with the *am*^{2} allele.

There was also the possibility that strains carrying the *sp* allele had a selective advantage on the sorbose medium used for plating, and this might account to some extent for the excess of *sp* among the wild types. Therefore, ascospores from the two types of cross mentioned above were plated both on medium containing sorbose and medium containing only 1.5 percent sucrose as the energy source. It is considerably more trouble to isolate *am*$^+$ from *am*^{2} × *am*^{3} crosses when the ascospores are plated on medium not containing sorbose, but it can be done satisfactorily. Comparison of the results from the two types of plating should show if, in fact, the presence of sorbose affects the kind of recombinant recovered. The results from these crosses, in which the experimental details were the same as those described for the first series of experiments, are given in Table 1.

It can be seen from Table 1 (lines 7 to 10) that, unlike the first series of crosses, there is no excess of those marker alleles, which were put into the cross with the am^2 allele, in the am^+ progeny. Thus, the possibility that the segment of genetic material carrying the am^2 allele was preferentially included in the formation of the am^+ recombinants in these crosses is discounted.

Inspection of the results, lines 7 to 10 in Table 1, shows that there was little difference between the frequency of am^+ carrying the *sp* allele on media with or without sorbose. In all four types of plating the ratio of *sp* to sp^+ is about one; the total numbers were nine *sp* to nine sp^+ on sorbose media, and 13 *sp* to 12 sp^+ on media without sorbose. Whatever the reason for the excess of the *sp* allele found in the first series of experiments, it seems clear from the later results that it was not due to a selective advantage of *sp* on sorbose medium.

The frequency of am^+ formation varied considerably between replicate experiments and between experiments which differed in the type of parents or kind of media used. The mean frequency for each cross and the total mean frequency are given in Table 1. The total mean frequency, 19.11 per 10^6 ascospores, is the best estimate available of the frequency of am^+ production. Using this estimate a 95 percent confidence interval was calculated for the sample obtained from each kind of cross. All of the mean frequencies given in Table 1 lie within their appropriate 95 percent confidence interval. That is, no combination of genotypes or type of media tested resulted in a frequency of am^+ production significantly different, at the five percent level, from the total mean frequency.

Genetic analysis of am^+ *recombinants from marked* $am^2 \times am^3$ *crosses*: The phenotypes of all the am^+ recombinants from these crosses were known accurately, and since Neurospora ascospores are normally haploid, the phenotypes should have been a direct reflection of the genotype. But it was considered possible that the am^+ were perhaps the result of rare meiotic abnormalities which gave rise to polyploidy, aneuploidy or some situation in which an extra piece of chromosome material carrying the *am* locus was present in the apparent am^+ nucleus. It is known that disomics occur in *N. crassa* (PITTENGER 1954), and disomics carrying certain combinations of *am* alleles do, in fact, have a more or less normal phenotype. But it is established (PATEMAN and FINCHAM 1958) that am^2 and am^3 do not give rise to the usual kind of phenotypically wild type disomic (pseudowild). The only practicable way of testing for some chromosomal abnormality in the am^+ wild types from $am^2 \times am^3$ crosses was to see if they were true breeding for am^+, and whatever allele was apparently carried at the spray and inositol loci. Therefore, a number of am^+ which had been first backcrossed to an *am* parent and reisolated, were crossed to appropriate tester strains and large numbers of ascospore progeny screened.

A total of 17 am^+ strains derived from marked $am^2 \times am^3$ crosses were crossed to stock wild types. The resultant progeny were plated and screened for *am* ascospores. The number of ascospores screened from each cross ranged from 4,700 to 38,100, estimated by the sampling method previously described. There were no *am* ascospores recovered in the total of 275,000 ascospores from the 17 crosses. Eight *sp* am^+ strains derived from marked $am^2 \times am^3$ crosses were crossed to *sp*

tester strains. The progeny were plated and screened for sp^+ ascospores. No sp^+ were observed in a total of 132,800 ascospores from the eight crosses. Six am^+ *inos* strains derived from marked $am^2 \times am^3$ crosses were crossed to *inos* tester strains. The progeny were plated and screened for $inos^+$ ascospores. No $inos^+$ were observed in a total of 108,600 ascospores from the six crosses.

These results show that a sample of 17 out of the 90 am^+ recombinants from marked crosses were true breeding for the am^+ allele, eight were true breeding for the *sp* allele and six for the *inos* allele. It seems clear that the majority of the wild type recombinants must be truly haploid for both the am^+ allele and those marker alleles which are expressed phenotypically. The data exclude at the 95 percent probability level that more than 16.25 percent of the am^+ progeny from marked $am^2 \times am^3$ crosses could have been the result of chromosome abnormalities.

The distribution of the outside markers in the two series of experiments with marked *am* × *am* crosses was very different (see Table 1), and at present no explanation can be suggested for this difference; but, in one important respect, all the experiments were similar. They all showed a great excess of strains which were parental in type with respect to the alleles at the *sp* and *inos* loci. In Table 2 the data are arranged to illustrate this. In each of the four possible kinds of marked *am* × *am* cross there were more parental types than those showing recombination for the outside markers. The total for all the crosses is 59 parentals to 31 recombinants. On the classical model of crossing over, all the parental types represent double crossovers, and this is a case of extremely high negative interference.

TABLE 2

Classification of am$^+$ *with respect to the distribution of the markers* sp *and* inos

Cross	Parental	Recombinant
sp^+ am^2 *inos* × *sp* am^3 $inos^+$	16	8
sp am^2 $inos^+$ × sp^+ am^3 *inos*	17	6
sp^+ am^2 $inos^+$ × *sp* am^3 *inos*	11	6
sp am^2 *inos* × sp^+ am^3 $inos^+$	15	11
Total	59	31

DISCUSSION

The production of wild type recombinants in crosses between alleles at the *am* locus is of particular interest since the wild types so formed regain the full normal ability to synthesize the enzyme glutamic dehydrogenase (PATEMAN and FINCHAM 1958). Preliminary work suggested that the origin of wild type recombinants could not be satisfactorily explained on the basis of classical crossing over theory (PATEMAN and FINCHAM 1958). Unfortunately, the frequency of such wild type production, about 19 per 10^6 live ascospores, is far too low for tetrad analysis. However, it was hoped that crosses between strains carrying marker alleles on either side of the *am* locus would help to elucidate the situation. The marked crosses established two main points.

If the am^+ were the result of classical crossing over, then in each cross about 90 percent of the am^+ progeny should have carried a particular combination of the outside markers. In the complementary cross, 90 percent of the am^+ should have carried the complementary combination of markers. There was no such obvious complementary pattern of distributions of the marker alleles in the am^+ progeny of complementary $am^s \times am^s$ crosses.

In the am^+ progeny of all the four possible kinds of marked $am^s \times am^s$ crosses, there was a considerable excess of apparent double exchanges in the region between the markers. If the 90 am^+ were the result of a single exchange between am^s and am^s then, assuming no interference, only seven to 11 percent of the am^+ would be expected to carry a further exchange in the *sp–inos* region. In fact, about 65 percent of the am^+ were apparent double exchanges with respect to the markers. It was not possible to estimate the excess of apparent exchanges on either side of the *am* locus, since the order of am^s and am^s within the locus is not known. The data provide no information concerning the production of reciprocal recombinants, since these presumably would have been of the *am* phenotype if formed, and consequently not detected.

An excess of apparent double exchanges, usually called negative interference, is now a well-recognized phenomenon in a variety of organisms. It has been reported in bacteriophage (STREISINGER and FRANKLIN 1956; CHASE and DOERMANN 1958); in Aspergillus (PRITCHARD 1955; CALEF 1957); in Neurospora (FREESE 1957; DE SERRES 1958), to mention only some of the reported cases. It is of interest that the intensity of negative interference, with apparent doubles more numerous than apparent singles, observed at the *am* locus is considerably greater than that usually observed in fungi. The results are not so easily compared with those obtained in bacteriophage, but the degree of negative interference reported above is probably of the same magnitude if not higher than that found in bacteriophage (CHASE and DOERMANN 1958).

In order to account for the many instances of high negative interference and of aberrant tetrad ratios in the literature, a "switch" hypothesis (FREESE 1957; CHASE and DOERMANN 1958; DE SERRES 1958) has been proposed. It is suggested that exchanges between homologous chromosomes can only occur in short "effective pairing" segments, and that such exchanges result from the new genetic

material using first one parental homologue as a template, then switching to the other, sometimes more than once. The average frequency of such "switches" is greater than one per effective pairing segment, and the process need not be reciprocal. This theory is sufficiently general to account for most of the known cases of recombination, and provides the most satisfactory explanation at the moment of this data concerning the *am* locus. In fact, the great excess of apparent double exchanges in crosses between *am* alleles is in accord with one of the tenets of this theory. Namely, the closer the mutational sites, the more marked should be the clustering of exchanges and, of course, the *am* alleles do show a very low frequency of recombination.

SUMMARY

1. In crosses between strains carrying the alleles am^2 and am^3 at the "amination deficient" locus in *Neurospora crassa*, am^+ recombinants are found in the progeny with a frequency of about 19 per 10^6 live ascospores.

2. The markers spray and inositol are adjacent to and on either side of the *am* locus. The four possible kinds of crosses between strains carrying different *am* alleles and the outside markers in different combinations were set up. Crosses which were complementary with respect to the markers carried by the parent strains did not show the expected complementary pattern of distribution of the markers in their progeny.

3. In crosses between marked am^2 and am^3 crosses, there was a great excess of apparent double exchanges, i.e., very high negative interference. In a total of 90 am^+ progeny from marked $am^2 \times am^3$ crosses, 59 possessed a parental combination of outside markers and 31 were recombinant. The expected number in the parental class, from a total of 90, was about nine.

4. It was shown that 17 am^+ strains from marked $am^2 \times am^3$ crosses were true breeding for the am^+ allele, eight were true breeding for the spray marker and six were true breeding for the inositol marker. The data exclude at the 95 percent probability level that more than 16.25 percent of the am^+ progeny from marked $am^2 \times am^3$ crosses could have been the result of chromosome abnormalities.

5. The results described are not explicable on the classical theory of crossing over, but are in reasonable accord with the "switch" theory of recombination.

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A STUDY OF IRREGULAR QUADRUPLETS IN SACCHAROMYCES

HAIG P. PAPAZIAN[1] AND CARL C. LINDEGREN

University of Southern Illinois, Carbondale, Illinois

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AN attempt will be made to introduce the term quadruplet or quad for the four cells produced by meiotic division or the cultures derived therefrom. The term "tetrad", then, defines its original object: the chromosomes at a certain stage of meiosis.

The fact that genes do not always segregate 2:2 in a quad has been confirmed both in microorganisms (PONTECORVO 1958) and higher plants (REIMANN-PHILIPP 1955). Whereas the first papers that drew attention to this phenomenon (LINDEGREN 1953) were mainly concerned with the elimination of explanations in terms of polyploidy or other classical phenomena, more recently attention has been focused on correlations with crossing over (STADLER 1959).

In the present study irregular segregation, or conversion, and crossing over of four linked genes have been analyzed in four different crosses or families. These crosses also included several other markers with which we are not here concerned.

Crosses were made by mixing two haploid populations of opposite mating type and then isolating and dissecting asci at room temperature. This allows little, if any, division of diploid cells before sporulation.

All four markers in all four families were linked, having a Parental:Non-parental ditype ratio greater than 1:1 at a one percent level of significance.

RESULTS

The genes *ch*, *hi*, *is* and *an*, requiring the amino acids choline, histidine, isoleucine and anthranilic acid, respectively, were examined in Family Nos. 99, 106, 107 and 108. Other mutants in these crosses are described in LINDEGREN, LINDEGREN, SCHULT and DESBOROUGH (1959). Mating media and dissection technique are described in LINDEGREN 1949 and media for testing requirements in MIDDLEKAUFF, HINO, YANG, LINDEGREN, and LINDEGREN 1956.

The best order of the four genes is the same in all four families; recombination distances with confidence limits at a five percent level are indicated in Figure 1. Confidence limits were calculated using half the number of spores to allow approximately for the correlation between spores of a quad (PAPAZIAN 1952).

The complete description of each quad containing a converted gene is given in Table 1. In order to compress this information and entertain the reader, a simple code has been used.

A description of a quad consists of a matrix of four rows for the four spores

[1] Present address: 169 Cold Spring Street, New Haven, Conn.

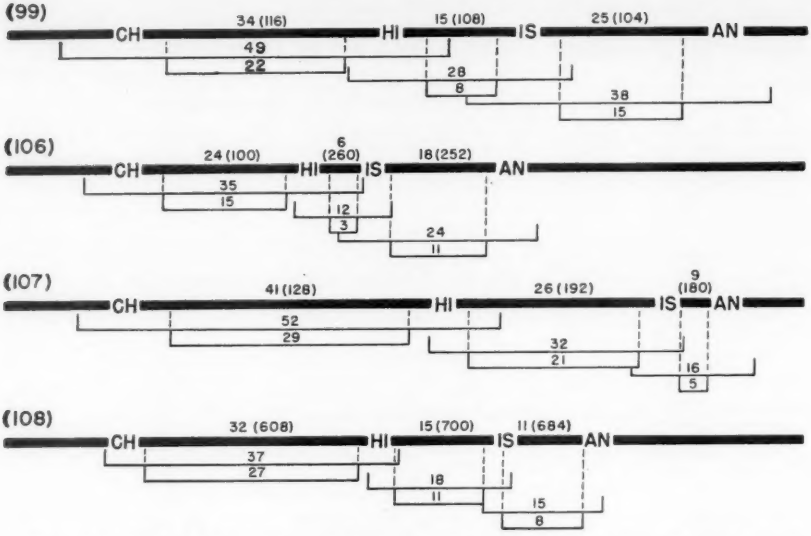

FIGURE 1.—Maps showing intervals between four genes in four families. Figures between letters give percentage recombination followed by total number of spores in brackets. Below are five percent confidence limits of each interval. The centromere lies to the left of *CH* (LINDEGREN *et al.* 1959).

TABLE 1

*Irregular quads and their crossover patterns**

	Quad no.	Segregation	Conversions	Simplest crossovers
#99	$P_0 = +\ hi + an$ (*ur, arg, a*)			
	$P_1 = \overline{ch + is +}$ (*th, cu, my, mg, dx, pa, act, a*)			
	21–696	1114	*AN* 1.	| / 5
	704	1446	*is* 4.	5 / 1
	708	1156	*hi* 4, *an* 1.	3 / 5
	716	1152	*hi* 3.	3 5
	720	1222	*IS* all.	1
	744		(*CH, HI, IS, AN*) all.	
	768	1112	*AN* 1.	5
	772	1555	*is* 3.	1
	776	1226	*hi* 4.	1 / 5

TABLE 1—Continued

*Irregular quads and their crossover patterns**

	Quad no.	Segregation	Conversions	Simplest crossovers
#106	$P_0 = +\ hi + an$ (*ur, cu, α*) $P_1 = \overline{ch + is +}$ (*t, dx, a*)			
	22–175	1155	*CH* 3.	3 | / |
	183, 439	1114	*CH* all.	| / | 5
	187	1155	*CH* all.	3 | / |
	199, 247	1113	*CH* all.	| / 5 |
	203	1113	*CH* 4.	| / 5 |
	207	1113	*CH* 3.	| / 5 |
	211, 311	1426	*CH* 3.	5 | 3 / | 1
	215, 367, 387, 395, 411, 423, 455, 459	1111	*CH* all.	| / |
	239	1144	*CH* all.	| / | 3
	255, 271, 463, 471	1444	*CH* 3.	| / | 1
	259, 299, 399, 431, 435, 443	1111	*CH* 3.	| / |
	291, 351	1115	*CH* 3.	5 | / |
	307	1666	*CH* 3, *HI* 3.	| 2 / 1 |
	319	1444	*CH* 3, *IS* all, *an* 3.	| / | 1
	331	1111	*CH* 3, *IS* 4, *AN* 2.	| / |
	347	1144	*CH* 3.	| / | 3
	355, 475	1446	*CH* 3.	5 | / | 1
	391, 415	1121	*CH* all.	| 3,5 / |
	403	1114	*CH* 3.	| / | 5
	419	1112	*CH* all, *IS* 3.	| 5 / |
	427	1112	*CH* all.	| 5 / |
	451	1111	*CH* all, *HI* 2, *AN* 2.	| / |

TABLE 1—Continued

*Irregular quads and their crossover patterns**

	Quad no.	Segregation	Conversions	Simplest crossovers
#107	$P_0 = +\ hi\ is\ an$ (*arg, ur, my, mg, me, dx*) $P_1 = \overline{ch + + +}$ (*g, ma, th*)			
	22–537, 639	1111	*CH* 3.	|
	553, 603	1444	*CH* 3.	| 1
	565	1111	(*CH, IS, AN*) all.	....
	635	1422	*CH* 3.	| 3 | 1
	643	1144	*CH* 3.	| 1
#108	$P_0 = \overline{ch + + +}$ (*th, as, a*) $P_1 = +\ hi\ is\ an$ (*arg, ur, pa, cu, a*)			
	22–719	1122	*IS* 4, *an* 1.	| 3
	727	1222	*is* 1.	| 1
	743	1233	*AN* 1.	3 | 1 | 4
	755	1222	*AN* 4.	| 3
	779	1462	*CH* 2.	3 | 5 | 1
	783	1116	(*HI, IS*) all.	| 1 3 |
	803	1221	*is* 3.	| 1, 5
	879	1344	*CH* 2.	1 | 3
	919	1555	*CH* 2.	1 |
	947	1336	*HI* 3.	| 5 1 |
	995	1111	*CH* 2, *HI* 2.	
	23–005	1111	*CH* 2, *HI* 4, *IS* 4, *AN* 4.	
	243	1553	*CH* 1.	1 | 5 |
	247	1152	*HI* 3.	3 | 5
	255	1111	*CH* 2, *HI* 3.	
	426	1222	*HI* 4.	| 1
	442	1111	*IS* 3.	
	406	1333	*CH* 2.	| 1 |

* Explanation in text.

and, in the present case, four columns for the four genes. The six possible orders of two alleles in each column of such a matrix have been given numbers as follows:

P_0	P_0	P_1	P_0	P_1	P_1
P_0	P_1	P_0	P_1	P_0	P_1
P_1	P_0	P_1	P_1	P_0	P_0
P_1	P_1	P_0	P_0	P_1	P_0
1	2	3	4	5	6

where P_0 represents the alleles derived from one parent and P_1 those from the other. The four columns of any matrix can then be represented by four numbers. Successive numbers represent the columns for genes *ch*, *hi*, *is* and *an* in that order.

The quads from yeast are unordered and therefore arbitrary; in the table the spore order in each quad has been arranged so that the first gene, *ch*, is in order 1.

Conversions are represented by two letters denoting the allele that is irregular and a number representing the row of the matrix in a 3:1 quad or the word "all" in a 4:0 quad.

The crossovers which can account for each quad are represented by a new convention, borrowed from games theory. In a two by two matrix the first and second columns represent the genetic strands 1 and 2, respectively, and the top and bottom rows represent strands 3 and 4, respectively. Thus, a crossover between strands 2 and 3 is represented by a figure in the top right-hand quadrant. To identify the interval in which a crossover occurs two numbers are reserved for each: 1 and 2 for the first interval, 3 and 4 for the second, 5 and 6 for the third. Quad 21708 entered as "1156 *hi* 4, *an* 1, $\begin{array}{l}3\,|\\ \hline |\,5\end{array}$" is:

CH	*hi*	*is*	(*an*)	and the crossover pattern is:	*CH*	*hi*	*IS*	*an*
CH	*hi*	*IS*	*AN*		*CH*	*hi*	*IS*	*an*
ch	*HI*	*IS*	*an*		*ch*	*HI*	*is*	(*an*)
ch	(*hi*)	*is*	*an*		*ch*	(*hi*)	*is*	*AN*

where the converted genes are in brackets.

It will be noticed that two-strand double crossovers are represented by figures in the same quadrant, i.e., in both the same row and column; three-strand doubles by figures in either the same row or column; four-strand doubles by figures in neither the same row or column.

Only four-spored asci were used in this work. three-spored asci accounted for less than five percent of the total asci. Two-spored asci were very rare, and no single-spored asci were seen. The number of asci dissected in Families 99, 106, 107 and 108 were 34, 94, 120 and 188, respectively. The asci in which one or more spores failed to germinate amounted to 9, 20, 42, and 23 percent, respectively. The genotype of the ungerminated spore, where the three others are known, has been inferred for the purpose of map distances in Figure 1.

Discrepancies in the total spores shown in Figure 1 and in the total quads which

can be inferred from Table 2 are accounted for by the cases in which cultures were lost or could not be classified. Loci with 4:0 segregation were not used for estimating map distances in Figure 1.

DISCUSSION

It is assumed that the conversions enumerated were not due to merely phenotypic changes or due to errors of classification but represent genotypes. This assumption is partly justified by the detailed analysis of similar conversions (LINDEGREN 1953).

Two genetic models are poor candidates to account for these conversions. The first is polyploidy or polysomy; this can give 3:1 or 4:0 quads and with two crossovers can allow genes on either side to segregate regularly; it cannot account for four recessives and no dominants in a quad, but we do not have such in the present data. If we assume that single crossovers are more frequent than doubles in the intervals under consideration, then this model would predict that more often than not a series of adjacent linked genes would be converted simultaneously. This is not the case in the present data (see below). Again it can be shown that if trisomy is used to account for single conversions, then the strand bearing the conversion will also be a crossover strand. Furthermore, spores from irregular quads would tend to be polysomic themselves and have irregular quads in their progeny. These conditions, as shown below, do not fit the present data.

The second model is somatic crossing over during division, if any, of the diploid cell. This could account for 4:0 or 3:1 quads depending upon the multiplicity of the chromosomes when crossing over takes place. Two crossovers could give conversions with regular segregation of genes on either side, but again if single crossovers are more frequent than two in adjacent intervals, then simultaneous adjacent conversions should be more frequent than single conversions.

In quads 21744 (*ME* segregated regularly), 22307, 22331, 22451, 22565, 22719, 23005 and 23255 an allele of one parent was in excess at one locus and an allele of the other parent was in excess at another locus. Such an occurrence is impossible on a trisomic model and with somatic crossing over three or more crossovers would be required. In balanced tetrasomics the allele in excess must always be the dominant which contradicts quads like 22719.

The frequencies of conversion are given in Table 2. The four genes in each

TABLE 2

*Frequencies of conversion**

Family	*ch* ⇄ *CH*	*hi* ⇄ *HI*	*is* ⇄ *IS*	*an* ⇄ *AN*	Total
99	2 = 1.7%	2–3 = 4.6%	4–2 = 5.5%	4–1 = 4.8%	18 = 4.0%
106	61 = 22.3%	2 = 0.7%	4 = 1.5%	2–1 = 1.0%	70 = 6.7%
107	8 = 8.0%	0	2 = 2.0%	2 = 2.0%	12 = 3.0%
108	8 = 1.3%	9 = 1.3%	5–2 = 1.0%	3–1 = 0.6%	28 = 1.1%
Total	79 = 7.2%	16 = 1.2%	19 = 1.6%	14 = 1.1%	128 = 2.7%

* In each column the first and positive number is the number of genes converted to wild type and the second, negative, number, the number converted to the recessive allele. Both figures are considered positive in calculating percentages.
Marginal percentages represent average conversions per gene, per Family and the grand average which is the average percentage genes converted per total genes observed.

family and any particular gene in each of the four families is converted either to the dominant or to the recessive with a frequency lying, for the most part, between one percent and five percent. One notable exception is the gene *ch* in Family 106. Conversion to the wild type is more frequent than to the recessive with the exception of gene *hi* in Family 99.

If the distribution of quads with 0, 1, 2, 3 conversions is compared with a Poisson, there are too few quads with a single converted gene and too many with multiple conversions. This is not entirely due to the five quads with four conversions and the one quad with eight conversions. The effect is significant at a five percent level of probability but not at a one percent level.

The segregation of the quads from which the parents came does not seem to affect the rate of conversion. One parent of Family 108 was a spore from Family 107 in which only the gene *mg* was converted to wild type. The other parent was the first row of quad 21708 in which *AN* was converted to *an* and *HI* to *hi*, but only the former conversion was on the strand used as parent; yet Family 108 is not exceptional as regards either of these genes. One parent of Family 107 was the first row of quad 21716 in which *HI* was converted to *hi* but on a different strand.

Conversion and crossing over: There is no evidence in this data of a correlation between the occurrence of conversion and of crossing over in a quad. This can be seen in Table 3 where the sum of crossovers in all three intervals is expressed as a mean number of crossovers (chiasmata) per quad (meiotic division). The distribution of crossovers in Family 108, the only family large enough to warrant such analysis, agree with a Poisson both for regular and irregular quads. This shows, incidentally, that there is no chromosome interference over this region. In quads in which there is a crossover and a conversion, there is no marked tendency for the crossover to occur in an interval adjacent to the conversion. In quads in which there is a conversion and a crossover in an adjacent interval, the strand carrying the converted gene should be involved in the crossover in half the cases if the two events are independent. Quad 21708, illustrated above, is an example of two conversions and two crossovers in adjacent intervals without the same strand being involved in either case. In the present data there are altogether 29 quads with a conversion and an adjacent crossover, and in only six of these does the crossover involve a strand carrying a conversion. This is significantly different (at a one percent level) from one half, but the effect is almost entirely due to the numerous

TABLE 3

Comparison of crossovers in regular and irregular quads

Family	Total crossovers per quad, in quads with and without converted genes: With	Without	Significance (P)
99	1.5	1.5	....
106	1.3	2.4	0.025
107	0.7	1.5	0.21
108	1.3	1.1	0.35

quads, especially in Family 106, in which *ch* segregates irregularly. If these are removed, then the ratio becomes four out of ten, which is very near to one half.

In all the foregoing, the minimum number of crossovers that will account for the distribution of alleles has been taken. The same distributions could result from a more complex pattern of crossovers, and it is only through lack of information that the simplest pattern is assumed. This assumption may well be erroneous since there is some evidence (PONTECORVO 1958) that crossovers occur in clusters over small regions. This may account for the fact that in studies of conversion over very small intervals there seems to be a correlation with crossing over not found in the present data.

SUMMARY

From this data it can only be said that alleles, in the heterozygous state, mutate to or are replaced by other alleles at a rate much higher than the spontaneous mutation rate. In particular genes and particular families, for instance *ch* in Family 106, this can reach over 20 percent.

It would also appear that the change is not random in the way that mutation is, but that the new allele is the same as a parental allele. This is indicated in the present data by the fact that conversions to wild type are much more frequent than to the recessive, and it is supported by other data (PAPAZIAN 1958) in which conversion affected a multiple allele system and in which conversion was not random to any allele of the series but always to the particular allele present in the heterozygote. The data indicates that neither polyploidy, polysomy, or somatic crossing over is involved.

Regular meiotic crossing over appears to be an independent phenomenon not correlated in any way with conversion.

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MUTATIONS AFFECTING QUANTITATIVE TRAITS IN THE SELFED PROGENY OF DOUBLED MONOPLOID MAIZE STOCKS[1]

G. F. SPRAGUE[2], W. A. RUSSELL[3], and L. H. PENNY[2]

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MONOPLOIDS occur normally in maize with low frequency and may be identified in the seedling stage by means of an appropriate system of genetic markers (CHASE 1949). A small percentage of such monoploid plants may exhibit sectorial diploidization. When sectors include all or parts of the ear and tassel, some seeds are produced upon self-fertilization. Each plant arising from such a seed is presumed to be completely homozygous since it represents, in a double state, the chromosomal complement of a single gamete. Since the frequency of detectable mutations is, to a great degree, a function of the genetic uniformity of the experimental material and of the techniques used, it was felt that doubled monoploid stocks would provide excellent material for a study of mutations affecting quantitative traits in maize.

Some of the changes observed in the present study were qualitative and, on the basis of limited data, appear to result from changes at a single locus. These were of limited interest, and no detailed data on them will be presented. The changes of primary interest were those whose effects led to significant changes in one or more of the several quantitative attributes studied. Since there are definite limitations in the types of analyses that may be used in a study of quantitative changes, the term "mutation" will be used in a broad sense, with no implication as to the exact nature of the change involved.

MATERIAL AND METHODS

Monoploids were isolated from stocks representing diverse genetic origins ranging from long-time inbred lines to synthetics. Seeds resulting from the self-fertilization of the monoploid plants were used as source material for this study. Such seeds were planted the following season and the resulting plants self-pollinated. The following year a progeny row was grown, representing each self-pollinated ear. Self-pollination was continued, and at harvest time two siblings were saved from each progeny row. This procedure was continued through successive generations. No conscious selection was practiced in any generation, ex-

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[2] Principal Agronomist and Agronomist, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, respectively.

[3] Associate Professor, Iowa State University.

cept for the one necessary restriction that each selfed ear saved have a minimum of 150 kernels. If a monoploid plant is designated as S_0, the diploid progeny resulting from self-pollination of such a plant as S_1, etc., this study covered generations from S_3 through S_6. Seeds produced in each generation were dried, shelled, and placed in cold storage until needed.

Qualitative changes in phenotype are directly observable. Quantitative changes are less readily recognized. All direct comparisons were made in replicated plantings to ensure that observed variation had some genetic basis. The material available for study was assigned to experiments on the basis of S_2 origin. Each experiment, with two minor exceptions, consisted of progenies from the parental S_2, the two S_3, the four S_4, and the eight S_5 ears tracing to a single S_2 plant. Using the remnant seed of these parental ears, each item was planted in replicated ten-plant progeny rows. The field design was a randomized block with ten replications. Eleven such progeny sets were grown, each as a separate experiment. Individual plant records were obtained for eight attributes in each experiment. In addition, individual plant records for date of silking were taken in three of the 11 experiments.

Stands were good, but not entirely uniform. A mean was calculated for each plot to minimize unequal frequencies, and an analysis of variance was performed on the plot means. An analysis of means would be expected to indicate significant differences if a mutant form had become fixed in the homozygous condition. It also might be possible to obtain a significant difference in means from comparisons between normal and segregating progenies if the mutant form represented a relatively large change. Significant differences were ignored unless they were consistent with the hierarchal structure of the experiment. With this restriction, only changes resulting in significant differences between parent and progeny or between siblings within a given generation were considered as representing genetic changes.

Significant differences arising through chance would be expected to be distributed at random among the 105 comparisons possible among the 15 entries comprising a single experiment. Of such comparisons only 21 are consistent with the parent-progeny or between-sibling relationships imposed by the hierarchal structure. Therefore, the great majority of significant differences arising from chance should be inconsistent with the hierarchal structure. Actually the majority of the significant differences observed were consistent with genetic expectations indicating that chance differences could play no more than a minor role in the present study.

Changes for which segregation was still occurring would be expected to increase the "within plot" variances. Variances were calculated for each plot, and an analysis of variance of these variances was made. Environmental variability was so great, however, that few of the analyses indicated significant differences. This approach was not pursued further, and thus, the only valid estimate of genetic change presented was that provided by the analysis of plot means.

For purposes of calculating mutation frequency, it was assumed that the only mutations that could be detected were those that had advanced to the equivalent

of an F_3 generation and that had become homozygous for the phenotypic change. In the majority of the experiments, this limited mutation frequency estimates to the gametes involved in the production of the parental S_1, S_2, two S_3 and four S_4 plants—or a total of 16 gametes. If the possibility of detecting a segregating progeny were accepted, the number of gametes tested per experiment would be increased to 32. An illustration of the form of the analysis of variance used is illustrated in Table 1. The variability associated with "entries" may be partitioned in a number of ways. Since the item of primary interest was the variation among progenies, the partition illustrated was chosen. The t test was used for estimating the significance of differences between the means using replication × entries as an estimate of σ_e^2.

EXPERIMENTAL RESULTS

It appears unnecessary to present detailed records of entry means for each attribute in each experiment. Typical results are presented in Table 2. Significant differences inconsistent with the hierarchal structure were ignored. In the data on ear length presented in Table 2, entry 120 had a mean ear length of 114.5 mm. This value differs significantly from entries 100 and 110. However, the sibling progenies of this ear grown as entries 121 and 122 were not significantly different from entries 000 or 100. Significant differences of this pattern might be attributed to the vagaries of sampling and were not counted as mutations. If it is assumed that a significant difference was conditioned either by several genes or by the effect of a heterozygote, then reversals of the type suggested by contrasts of entry 120 with its parent and progeny could have a logical explanation. Entry 222 differed significantly from its parent 220 and was considered as representing one mutational change. This procedure introduces a possible bias, since performance records were not available for S_7 progenies. It would appear, however, that this bias would not materially exceed five percent.

TABLE 1

An illustration of the form of analysis of variance used for each attribute in the several experiments. (Ear length mm, experiment 80)

Source of variation	d.f.	m.s.
Replications	9	128.19
Entries	14	166.10
Parents *vs.* progeny		
S_3 *vs.* S_4 mean	1	58.12
S_4 *vs.* mean of S_5's within S_4's	2	172.34
S_5's *vs.* mean of S_6's within S_5's	4	221.21
Between progeny		
Between S_4's	1	38.09
Between S_5's within same S_4	2	185.26
Between S_6's within same S_5	4	160.46
Replication × entries	126	60.50
Total	149	

TABLE 2

Representative data indicating the type of parent-progeny differences that were interpreted as mutational changes

Coded pedigree	Generation	Experiment 81 Ear length (mms)	Experiment 81 Leaf width (cms)	Experiment 81 Date of silking after July 1	Experiment 83 Weight of shelled grain (gms)	Experiment 89 Plant height (cms)
000	S_3	122.8	11.9	24.5	53.2	123.2
100	S_4	127.2	11.9	24.7	41.8	122.8*
200	S_4	124.5	12.1	24.8	43.7	121.2†
110	S_5	122.6	11.7*	24.6*	43.3*	96.2*
120	S_5	114.5	11.7	25.1	44.8	120.7
210	S_5	125.8	12.0	24.4	57.4†	123.3
220	S_5	122.9*	12.0	24.5	47.2	115.0†
111	S_6	120.1	11.4*	25.6*	30.6*	102.3
112	S_6	129.3	11.7	25.7*	40.9	102.8
121	S_6	124.8	11.8	25.4	29.3	119.6
122	S_6	119.4	12.1	24.6	34.8	122.2
211	S_6	125.1	11.9	24.8	70.3†	118.4
212	S_6	124.8	11.9	25.3	57.9	122.2
221	S_6	127.0	12.2	24.9	35.4	115.0
222	S_6	130.9*	12.1	24.9	36.8	117.2
C.V.		6.03	2.91	3.48	29.71	6.44
L.S.D. (.05)		7.10	0.30	0.77	11.70	6.70
No. of mutations		1	1	1	2	2

* † Items marked by a common superscript represent significant parent-progeny differences each of which was interpreted as a single mutation.

TABLE 3

Total number of mutations per attribute for each of the 11 families (experiments)

Expt. no.	Plant height	Leaf width	No. of tassel branches	No. of kernel rows	Ear length	Ear diameter	Wt. per 100 kernels	Wt. of shelled grain per plant	Date of silking	Total of experiments
80	1	1	1	0	1	1	0	2		7
81	2	1	1	2	2	1	2	1	1	13
82	1	1	2	0	1	0	0	1	2	8
83	2	2	0	0	1	1	1	2	.	9
85	1	1	2	0	1	0	1	0	.	6
86	1	0	0	1	2	0	0	0	.	4
87	1	2	1	0	0	0	0	1	.	5
88	0	1	0	0	2	0	0	0	.	3
89	2	2	3	3	2	2	4	2	2	22
90	1	0	1	1	0	1	0	0	.	4
91	2	2	2	1	2	0	0	0	.	9
Total	14	13	13	8	14	6	8	9	5	90

Using the criteria just described, the total number of significant changes (mutations) for each attribute in each experiment was calculated. The data are presented in Table 3. The most striking characteristic of these data is the large number of mutations. As mentioned previously, the procedure used estimates mutations in only eight plants or 16 gametes per experiment (16 plants or 32

gametes if segregating progenies are assumed to produce a recognizable effect). Experiments 85 and 89 were exceptions to this rule. Experiment 85 involved five and experiment 89 involved 11 tested plants. An average for all experiments indicates a mutation rate of approximately six mutations detected per attribute per 100 gametes tested.

With quantitative traits, it is conceivable that one mutation might affect more than a single attribute. This possibility was explored by comparing the time of detection and the subsequent distribution pattern of all possible pairs of presumed mutations. When this was done, the number of independent mutations was reduced by approximately 25 percent. The correspondence of distribution does not constitute proof of pleiotropism, but it appears desirable to accept the more conservative figure of 4.5 mutations per attribute per 100 gametes tested.

If a mutation rate, u, is assumed, then the probability that a haploid having sectors of diploid tissue in the male and female inflorescence or a homozygous diploid will give rise to heterozygous progeny through mutation at a single locus is equal to $2u$. Since all progenies were perpetuated by self-fertilization, any heterozygosity arising by mutation would be halved in each succeeding generation. Since new mutations could arise in each generation, the total heterozygosity at any generation could be expressed as $2u\ (1 + \frac{1}{2} + \frac{1}{4} + \frac{1}{8} + - - -) = 4u$, as shown by HALDANE (1936). The frequency of heterozygous plants could not be determined accurately in these experiments; thus, this method could not be used for estimating the mutation rate. The only estimates of mutation rates that could be obtained were those based upon the number of significant changes presented in Table 3.

The estimated value, 4.5 per attribute per 100 gametes, cannot be interpreted in terms of mutations per locus without a considerable number of simplifying assumptions. The necessary assumptions appear to be so unrealistic that calculated values have little significance. They do, however, suggest either that the mutation rate is substantially greater than the commonly accepted figure, 10^{-5}, or that the number of loci is substantially greater than previous estimates of 5000 (CROW 1948).

Additional evidence supporting mutation as the mechanism causing increased genetic variability can be obtained from the analysis of variance, particularly the three "within generation" estimates. If the significant differences among siblings or between parent and progeny were solely the result of some form of structural hybridity arising in the haploid parent, the between-progeny variances with self-fertilization would be expected to decrease in subsequent generations according to the series $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$ $- - -$. Data bearing on this point are presented in Table 4. The values presented are estimates of genetic variance averaged over the 11 experiments. The variance estimates for the individual experiments were obtained from the relationship $\sigma_g^2 = \sigma_p^2 - \sigma_e^2$, where σ_g^2 is the genetic variance, σ_p^2 is the mean square variance for the appropriate "between-progeny" component, and σ_e^2 is the error variance. Contrasts between the magnitude of between S_4's *vs.* S_6's within S_5 are available from 83 analyses. In 11 of the comparisons the estimates were equal. In 53 of the remaining 72 cases the S_6's within the S_5 estimates were greater. This would appear to rule out structural hybridity as an

TABLE 4

Average estimates of genetic variance for the three "between progeny" partitions for each attribute studied

Attribute	Between progeny variances		
	S_4 within S_3	S_5 within S_4	S_6 within S_5
Plant height	10.69	51.31	69.79
Leaf width	—1.83	4.75	—0.64
Number of tassel branches	0.51	98.71	4.44
Number of kernel rows	0.86	4.29	0.45
Ear length	25.97	48.65	12.53
Ear diameter	0.10	0.58	0.62
Weight per 100 kernels	1.44	3.29	3.53
Weight of shelled grain	—17.79	79.26	47.98
Date of silking	—0.04	13.00	0.57

important factor and suggests that the process inducing variation must be a continuing one.

The very considerable variation among the estimates for the "between-progeny" components is a result of the small number of degrees of freedom. Thus, the mean square for each of the subdivisions is poorly estimated and would have a large associated standard error. Disregarding the algebraic magnitude, the frequency with which the difference between a particular "between" mean square was less than the error estimate suggests a definite trend. The percentages of negative estimates for between S_4, S_5's within S_4, and S_6's within S_5 were 59.0, 37.4, and 23.1, respectively. A more detailed statistical treatment of the available data appears unwarranted. The analysis used indicates that genetic variability increases with continued self-fertilization. More precise estimates of genetic variability must come from a different experimental approach. Seed preparation for such studies is currently underway.

Evidence for genetic change also is provided by crosses of S_5 lines within a family to their S_3 parent and by crosses of S_3 and S_5 lines to unrelated tester inbreds. The pertinent data for one such series of comparisons involving the attribute, number of tassel branches, are presented in Table 5. The F_2 distributions from crosses involving the S_5 lines HD 601-2-1-2 or HD 601-2-3-1 with their S_3 parent, HD 601-2, were significantly different, indicating that the two distributions could not be considered as random samples drawn from a single homogeneous population. When each of these three lines was crossed to either W92 or W17RB as a common tester parent, χ^2 was highly significant for each set of F_2 distributions.

In the W92 series the significance among the set of three results from the dissimilar performance of HD 601-2 and HD 601-2-1-2. The differences between HD 601-2 and HD 601-2-3-1 were not sufficiently large to be considered significant. However, when W17RB was used as a common tester, all differences were judged significant. Thus, in each of three comparisons HD 601-2-1-2 and

TABLE 5

F_2 distributions for a number of tassel branches in test crosses comparing related S_3 and S_5 lines from a single family

Test crosses	No. of tassel branches midclass values 2.5	6.5	10.5	14.5	18.5	22.5	26.5	30.5	Total no.	Mean
HD601–2 × HD601–2–1–2	..	2	31	43	58	56	21	12	223	18.95
HD601–2 × HD601–2–3–1	..	..	2	11	50	81	31	6	181	21.78
W92 × HD601–2	..	..	10	17	53	46	27	23	176	21.51
W92 × HD601–2–1–2	..	8	24	80	89	41	4	..	246	16.94
W92 × HD601–2–3–1	..	2	11	27	56	49	23	18	186	20.69
W17RB × HD601–2	4	18	39	62	41	24	..	..	188	14.65
W17RB × HD601–2–1–2	125	128	15	2	..	..	..	..	270	4.97
W17RB × HD601–2–3–1	83	127	42	13	6	3	..	..	274	6.73

HD 601-2-3-1 were shown to be genetically unlike. In one of two comparisons, HD 601-2-3-1 was indicated as genetically distinct from HD 601-2. The differences between 2-1-2 and 2-3-1 and between these and the parent form HD 601-2 must have been of recent origin. Some type of mutation appears to provide the best explanation for these observed differences. Dissimilar genotypes and modifier backgrounds may account for the differences observed between the two sets of crosses involving unrelated tester material.

Combining ability: The original S_3 line and one or more of its divergent S_5 generation derivatives were crossed to two long-time inbred lines, WF9 and B14, and the test crosses evaluated in replicated yield trials. The material comprising each family was grown as a separate experiment with each entry replicated ten times. Standard field plot techniques were used. The data are presented in Table 6.

A significant difference between S_3 parent and S_5 progeny was observed in two of the 38 contrasts. In these instances, the lower yield was associated with earlier maturity. The proportion of significant differences observed was no greater than might be expected on the basis of sampling. If more than a single genetic change would be required to modify yield performance at the hybrid level, the time period involved was too short to expect significant differences even though a high mutation rate might be involved. Furthermore, the only mutations that could be expressed in the test crosses would be those having some degree of dominance.

DISCUSSION

Attempts to obtain an estimate of mutation rate for loci conditioning quantitative traits involve a number of unwieldly problems. First, the methodology is cumbersome, and the testing of even a small population involves a very considerable expenditure of time and effort. Second, when changes are detected, a precise genetic analysis is not feasible. In spite of these and other limitations, the number

TABLE 6

Yield in bushels per acre for test crosses comparing original S_3 lines and one or more of their S_5 generation derivatives

Pedigree	Yield in bushels per acre when combined with WF9	B14	Average
HD698–1	89.2	89.5	89.4
HD698–1–2–1	85.1	89.3	87.2
HD698–1–3–2	85.0	87.7	86.4
HD601–2	77.2	85.1	81.2
HD601–2–1–4	83.1	82.9	83.0
HD601–2–1–6	77.1	75.6*	76.4*
HD601–2–3–6	80.0	89.4	84.7
HD601–6	70.5	81.2	75.8
HD601–6–1–2	76.7	83.6	80.2
HD513–3	92.5	81.8	87.2
HD513–3–1–1	97.3	84.1	90.7
HD513–5	88.1	86.5	87.3
HD513–5–3–3	85.3	87.5	86.9
HD510–1	82.0	85.2	83.6
HD510–1–1–1	84.6	87.5	86.0
HD510–5	75.9	82.3	79.1
HD510–5–1–2	80.1	80.3	80.2
HD510–9	79.0	84.0	81.5
HD510–9–2–2	81.2	82.1	81.6
HD502–2	80.3	67.9	74.1
HD502–2–1–1	89.7	68.5	79.1
HD502–2–1–2	85.1	68.5	76.8
HD502–2–2–1	86.8	69.3	78.0
HD502–2–2–2	85.7	69.5	78.1
HD502–8	89.0	68.9	79.0
HD502–8–2–1	88.6	70.2	79.4
HD502–8–2–2	85.9	68.6	77.2
HD73–1†	85.6	83.5	84.6
HD73–1–1–1	80.2	89.9	85.0
HD73–1–3–1	84.7	85.2	85.0
W22	82.8	87.0	84.9

* Yield difference significant at the 5 percent level.
† HD73 was derived from the inbred line W22.

of the significant genetic changes obtained in the present study is sufficiently great to warrant some speculation as to a possible cause.

Structural hybridity and mutable systems are known to have a marked influence on increasing the frequency of certain types of mutations. Meiosis in monoploids has been studied by FORD (1952), MCCLINTOCK (1953), and RANDOLPH

(1932). Pachytene preparations of monoploid plants exhibit varying degrees of apparent chromosome doubleness. This doubleness may arise from foldback areas within chromosomes or non-homologous association between chromosomes. If such non-homologous pairing gives rise to genetic crossing over, then the resulting gametes might possess structural dissimilarities. Zygotes derived from such gametes might be heterozygous for various types of chromosomal aberrations which could give rise to duplication-deficiency phenomenon in any progeny resulting.

However, the seeds used to initiate the present study had their origin in diploid sectors on the ear. Also functional pollen is shed from only diploid sectors of the tassel. Since diploid maternal tissue is involved in both cases, it is presumed that meiosis would be normal and that non-homologous pairing would be of little or no importance. While the material used in this study was not subjected to cytological analysis, there are additional reasons to feel that chromosomal aberrations could have played only a minor role in the observed changes. Reciprocal translocations would lead to semisterility, which was not observed. Duplications would lead to severe gametic competition and would rapidly be eliminated. All of the chromosomal deficiencies known in maize are lethal in the homozygous condition and most are haplo-lethal in the male gametophyte. Inversions, depending upon the size of the inverted segment, would lead to some reduction in fertility. Again, such types were not observed, but they could have been overlooked. The selection of seed ears that possessed a minimum of 150 kernels, as mentioned earlier, would impose some restriction against plants carrying a major chromosome aberration. Furthermore, the evidence presented in Table 4 indicates that the process involved is continuing and not limited to the monoploid phase. The testcross data in Table 6 provided no evidence for sterility and would appear to rule out any possibility of involvement of either homozygous inversions or translocations.

Several mutable gene systems have been reported for maize. For the most part these appear to be rather specific in their effects and to affect only a limited number of attributes. If a mutable gene system were postulated to account for the present results, it would have to have an effect on a number of different quantitative attributes; and each of the monoploids involved in this study must have carried such a system. This appears quite unlikely unless one further assumes that, in some manner, the monoploid condition is either directly or indirectly responsible for the origin or is the result of the mutable system. Any resulting instability would need to have its origin in something other than major chromatin rearrangements for the reasons just outlined. Furthermore, if such an explanation were valid, one would have to assume either that a large number of such changes was involved in each monoploid parent or that any of a large number of loci could have an important influence on each of the attributes studied.

The possibility that monoploidy may play some role in the origin of mutable systems is conceivable. The possibility that genetic instability may be widespread throughout the maize genome has been established by McCLINTOCK (1950, 1951, 1953), BRINK and NILAN (1952), and others. However, the mutable systems reported thus far have dealt with qualitative effects. One could argue

that if mutable systems are involved in the present study, they must represent a special class, since the increase in frequency appears to have been restricted to quantitative traits leaving qualitative effects not markedly in excess of normal expectation. This argument has definite limitations, since the parental material used in the production of monoploids carried relatively few genes that would have been satisfactory as markers. Furthermore, nothing is known as to the number of loci where mutational changes could produce an effect on a quantitative trait such as plant height. If a sufficiently large number of loci were assumed, then the apparent discrepancy between frequencies of changes affecting quantitative and qualitative traits might disappear.

The possible role of monoploidy as a causal factor in the origin of mutable systems can be evaluated only when comparable studies have been conducted with long-time inbred material. Seed preparation for such a study has been finished, but the field comparisons remain to be completed.

The estimates of frequency of genetic change provided by this study are subject to several types of bias. The restriction that any ear saved to propagate a line must have a minimum of 150 kernels would ensure the elimination of all lethals and sublethals. It would also reduce the estimates of frequency of occurrence for changes having a markedly depressing effect on yield level. The procedures used in the identification of genetic changes would preclude the detection of anything except major changes. For example, the data provide a strong suggestion of small stepwise changes that were not significant in any direct parent-progeny comparison but that were highly significant when S_3 *vs.* S_6 comparisons were made. Changes of this sort were ignored in the mutation frequency tabulations. Presumably, also, other changes that failed to achieve significance in the present study may represent real alterations and would have been judged significant had a larger number of replications been used.

In several instances, significant differences were observed between parent and progeny that were not retained in subsequent generations. These could be considered as chance deviations, segregation, or some form of reverse mutation. If the mutation rate is as high as the present data suggest, the possibility of reverse mutations or additional changes whose effects simulate reverse mutations cannot be ignored. However, in the present case significant differences of this sort were excluded from the tabulation. Other mutations may have gone undetected because of failure to become established in a homozygous condition.

Significant differences between parent and progeny, subject to the restrictions just outlined, were interpreted as representing a changed condition at a single locus. This would appear to be the simplest interpretation possible. However, there remains a possibility that the observed changes represent the culmination of a series of changes each having a smaller and nonsignificant effect. If this were true, each significant change might result from the multigenic alteration rather than a single locus effect. Each of these possibilities would lead to an underestimation of the true mutation frequency.

Since the individual changes were not tested for possible allelism, a possibility remains that a given mutation might be counted more than once. For example,

in one line of descent a difference in plant height might be apparent in one of two S_4 progenies. The other S_4 progeny could be heterozygous for the same mutation, and this change might be established as a distinct type in either the S_5 or S_6 generation. This may have been a factor in the present study. An attempt has been made, however, to minimize this possible bias by disregarding changes in different lines of descent which led to similar mean values. Thus two or more significant parent-progeny differences in S_5 or S_6 characterized by similar means were recorded as representing only a single mutation. Any duplications remaining would, of course, lead to an overestimation of the mutation frequency. It is the opinion of the authors that any net bias is in the direction of underestimation rather than overestimation.

If the mutation rate of normal inbred lines of corn is as high as these studies suggest, one might question how stability of appearance and performance could be maintained. It is a well-established fact that changes in phenotype do occur, since long-time inbred lines, when exchanged and maintained by different workers over a period of time and under different environments, become noticeably distinct. The degree of stability achieved is probably a direct result of the continuing selection practiced. In propagation of an inbred line, recognizable deviants would be avoided at pollination time unless they appeared to be of superior type. A second selection for typical plants would occur at harvest time. Thus, any mutations causing recognizable deviations from type would tend to be rapidly eliminated.

SUMMARY

Doubled maize monoploids would be expected to be completely homozygous since they arise from a single monoploid gamete. A detailed study was made of a series of doubled monoploids involving their S_3 through S_6 progenies. Significant differences between means were observed for each of the quantitative traits measured. Such significant differences were interpreted as resulting from some type of mutational change. The rate observed was 4.5 mutations per attribute per 100 gametes tested. The various factors that might influence the reliability of the observed mutation rate are discussed.

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STUDIES ON THE EXPRESSION OF VARIOUS SINGED ALLELES IN DROSOPHILA MELANOGASTER

HARVEY ALAN BENDER[1]

Department of Biological Sciences[2],
Northwestern University, Evanston, Illinois

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RECENT work on a set of alleles that pleiotropically affect both setal morphology and oogenesis has provided a complex of data that have not been systematically analyzed. These alleles constitute the sex-linked mutation singed. In 1951, IVES and NOYES demonstrated 0.04 percent crossing over between sn^3 and sn^{50k}. Genes such as these which are physically close together, similar in function, and yet separable by crossing over are called pseudoalleles. Position pseudoallelism (LEWIS 1942) was demonstrated among the singed mutants by HEXTER (1955) who showed that sn^3 +/+ sn^1 flies displayed a singed phenotype while sn^3 sn^1/ ++ flies were wild type in appearance. Subsequently he demonstrated three loci in the *sn* complex which he preliminarily characterized (HEXTER 1957).

Twenty-eight singed mutations have been reported since MOHR's first record (1922), but at present only 11 are available, and these were used in the following study. These stocks and progeny of the possible combinations were examined for bristle morphology, fertility, and the anatomy of the female reproductive tract. The classes of phenotypes were analyzed; this phenotypic array of gene effect was then used together with crossover data to provide a model for the loci involved.

MATERIAL AND METHODS

The *Drosophila melanogaster* stocks used in this investigation were supplied through the kindness of DRS. R. C. KING, W. HEXTER, I. I. OSTER, and D. F. POULSON. The history of the *Drosophila melanogaster* stocks utilized and their source is provided in Tables 1 and 2.

The flies in this study were grown on medium (corn meal-Karo-agar mixture) at 25°C in half-pint bottles kept in a refrigerated incubator. Observations of the setal morphology of 25 males and 25 females of each of the available *sn* mutants were made with stereoscopic microscope. Bristles were scored as "gnarled" if the anterior and/or posterior scutellar setae were knotted together, "kinky" if they were thickened or bent. Hairs were scored as "kinky" if any bending or kinking

[1] Present address: Department of Entomology and Parasitology, University of California, Berkeley, California.

[2] Prepared from a thesis submitted to the Graduate School, Northwestern University, in partial fulfillment of the degree, Doctor of Philosophy, 1959.

TABLE 1

Singed mutants

Singed mutant	Reproductive ability of homozygote	Discoverer	Date first observed	Occurrence	Stock maintained as	Source
sn^{1}	sterile	MOHR*	Oct. 5, 1918	Spontaneous	*sn*^{1}/M5	Amherst College
sn^{2}	fertile	BRIDGES*	Dec. 2, 1919	Spontaneous	*sn*^{2}	Amherst College
sn^{3}	fertile	MOHR*	July 11, 1922	Spontaneous	*sn*^{3}	Amherst College
sn^{4}	fertile	BRIDGES*	Jan. 26, 1930	Spontaneous	*sn*^{4}	Amherst College
sn^{3+4}	fertile	DUNCAN*	May 20, 1934	Spontaneous	*sn*$^{3+4}$	Amherst College
sn^{5}	sterile	BRIDGES*	Feb. 5, 1930	Spontaneous	*w sn*^{5}/M5	Amherst College
sn^{50k}	sterile	IVES and NOYES†	Nov., 1950	X-ray induced	*y sn*^{50k} *ras*^{2}/M5	Amherst College
sn^{36a}	sterile	SPENCER*	Jan. 21, 1936	Spontaneous	*sn*^{36a}/M5	Cal. Inst. of Tech.
sn^{22}	sterile	MULLER‡	.....	X-ray induced	*In(1)49 sn*^{22}/M5	Indiana Univ.
sn^{55a}	fertile?	HILLMAN§	Jan., 1955	Spontaneous	*y*^{2} *w*^{a} *cv sn*^{55} *v* +^{f}	Yale University
sn^{c}	sterile	MULLER‡	.....	Spontaneous	*sn*^{c}/ *y Hw In(1)49 m g*	Indiana Univ.

* BRIDGES and BREHME 1944.
† IVES and NOYES 1951.
‡ DR. I. I. OSTER (Personal communication).
§ HILLMAN 1958.

TABLE 2

Singed double mutants synthesized HEXTER (Amherst College)

Double singed mutant	Fertility of homozygote	Stock
sn^{1}*sn*^{2}	sterile	*cm ct*^{6} *sn*^{2}*sn*^{1}/M5
sn^{1}*sn*^{3}	sterile	*cm ct*^{6} *sn*^{3}*sn*^{1}/M5
sn^{1}*sn*^{4}	sterile	*cm ct*^{6} *sn*^{4}*sn*^{1}/M5
sn^{2}*sn*^{3}	sterile*	*ct*^{n}*sn*^{3}*sn*^{2}/M5
sn^{2}*sn*^{4}	fertile*	*ct*^{n}*sn*^{3}*sn*^{2}/M5

* See text.

could be observed. A wild type phenotype of the hairs denotes no visible bending or kinking.

Quadruplicate crosses were made in all the possible permutations of representatives of the three known pseudoallelic loci of the *sn* complex. Reciprocal crosses were made also. The results of these reciprocal crosses indicated no differences and consequently, the data were considered collectively. The morphology of the bristles and hairs of 25 females emerging from each cross was examined, and flies were placed in the categories described above. Flies were classified as fertile or sterile on the basis of the presence or absence of larvae in quadruplicate cultures where six freshly hatched females of the genotype to be tested were crossed to six freshly hatched males. Feulgen-stained whole mounts were made of the ovaries of the various singed mutants. The procedure for the preparation of Feulgen whole mounts was developed from KING, BURNETT, and STALEY (1957).

The stages of oogenesis as described by KING, RUBINSON and SMITH (1956) were utilized in this study.

RESULTS

Classification based on morphology of bristles and hairs

The available *sn* mutants can be classified into three categories on the basis of bristle and hair morphology: Class A—kinky hairs and gnarled bristles (sn^1, sn^3, sn^5, sn^{50k}, sn^1sn^2, sn^1sn^3, sn^1sn^4, sn^2sn^3, sn^{z2}, and sn^c); Class B—kinky bristles only (sn^2, sn^4, and sn^{34e}); Class C—gnarled bristles only (sn^{36a}). These observations support and supplement those of HEXTER (1957). Figure 1 demonstrates the typical appearance of wild type bristles and hairs. Figures 2 and 3 illustrate the typical appearance of the Class A, sn^1 and sn^3 mutants. Figures 4 and 5 illustrate the appearance of two Class B mutants sn^2 and sn^4. Figure 6 illustrates the phenotype of the Class C mutant sn^{36a}. It is interesting to point out that the double mutant homozygotes sn^1sn^4, sn^2sn^3 and sn^1sn^2 shown in Figures 7, 8, and 9 respectively, are indistinguishable from one another and from the homozygous Class A mutants.

The *sn* mutants can be classed into four categories if fertility data are used in addition to setal morphology: Class 1—sterile with kinky hairs and gnarled bristles (sn^1, sn^5, sn^{50k}, sn^{z2}, sn^c and the double mutants); Class 2—fertile with kinky bristles only (sn^2, sn^4, and sn^{34e}); Class 3—fertile with kinky hairs and gnarled bristles (sn^3); and Class 4—sterile with gnarled bristles only (sn^{36a}).

Phenotypic consequences of genotypic combinations of sn *mutants*

Fertility: It was found that any individual compounded with a fertile *sn* allele on one chromosome was fertile, and that an individual homozygous for any two sterile *sn* alleles was sterile (Table 3). However, individuals homozygous for the double mutant sn^2sn^3 are sterile; whereas individuals homozygous for sn^2 or sn^3 are fertile. The sterility of the sn^2sn^3 stock is puzzling since synthesis of a second sn^2sn^3 stock (HEXTER, personal communication) resulted in a highly fertile double homozygote.

Setal morphology: It was noted (Table 3) that Class 2 bristle and hair morphology is dominant over Class 3 and Class 4 and blends with Class 1. Class 4 bristle and hair morphology is dominant over Class 1 and 3.

Oogenesis in singed mutants

Cytological investigations: Oogenesis was studied in representatives of all members of all four classes of mutants and in representatives of most of the sterile progeny of all crosses among these four classes as listed in Table 3. The number of ovaries examined in each case is given in Table 4.

Two major types can be recognized: I. Normal oogenesis occurs in all fertile *sn* mutants; II. Abnormal oogenesis occurs in infertile *sn* mutants. The sterile category can be further subdivided on the basis of differences in their oogenesis

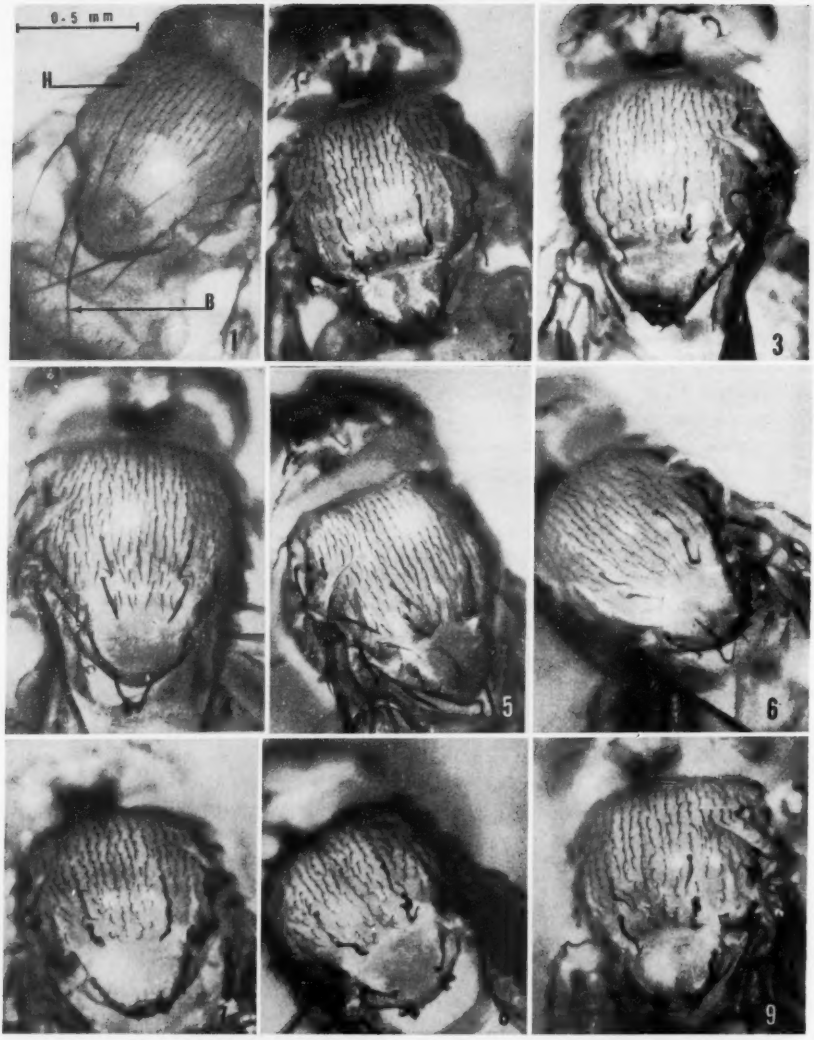


FIGURE 1.—The dorsal aspect of the thoracic region of a wild type *Drosophila melanogaster*. Note the bristles (B) (macrochaetae) and hairs (H) (microchaetae). FIGURES 2 and 3.—Class A singed mutants (sn^1 and sn^3). Note the gnarled bristles and kinky hairs. FIGURES 4 and 5.—Two class B mutants, sn^2 and sn^4. These mutants are typified by having kinky bristles and wild type hairs. FIGURE 6.—The Class C mutant sn^{36a}. Note the gnarled bristles and nonkinky hairs. Note also the hairs are somewhat intermediate to those of the Class A and wild types. FIGURES 7–9.—Three mutants homozygous for two singed alleles, (sn^1sn^4, sn^2sn^3, and sn^1sn^2 respectively).

TABLE 3

Crosses of all possible permutations of the three representatives of the singed pseudoallelic complex

	Class 1 *sn*^{1}	Class 2 *sn*^{2}	Class 3 *sn*^{3}	Class 4 *sn*^{3a}	*sn*^{1}*sn*^{2}	*sn*^{1}*sn*^{3}	*sn*^{2}*sn*^{3}‡
Class 1 *sn*^{1}	gnarled b.* kinky h.† Sterile	gnarled b. wild type h. Fertile	gnarled b. kinky h. Fertile	gnarled b. wild type h. Sterile	gnarled b. kinky h. Sterile	gnarled b. kinky h. Sterile	gnarled b. kinky h. Sterile
Class 2 *sn*^{2}	.....	kinky b. wild type h. Fertile	kinky b. wild type h. Fertile	kinky b. wild type h. Fertile	kinky b. wild type h. Fertile	kinky b. wild type h. Fertile	kinky b. wild type h. Fertile
Class 3 *sn*^{3}	.....	.....	gnarled b. kinky h. Fertile	gnarled b. wild type h. Fertile	gnarled b. kinky h. Fertile	gnarled b. kinky h. Fertile	gnarled b. kinky h. Fertile
Class 4 *sn*^{3a}	.....	.....	.....	gnarled b. wild type h. Sterile	gnarled b. wild type h. Sterile	gnarled b. wild type h. Sterile	gnarled b. wild type h. Sterile
sn^1*sn*^{2}	.....	.....	.....	.....	gnarled b. kinky h. Sterile	gnarled b. kinky h. Sterile	gnarled b. kinky h. Sterile
sn^1*sn*^{3}	.....	.....	.....	.....	.....	gnarled b. kinky h. Sterile	gnarled b. kinky h. Sterile
sn^2*sn*^{3}	.....	.....	.....	.....	.....	.....	gnarled b. kinky h. Sterile

* Bristles.
† Hairs.
‡ Sterile (see text).

TABLE 4

Ovarian preparations

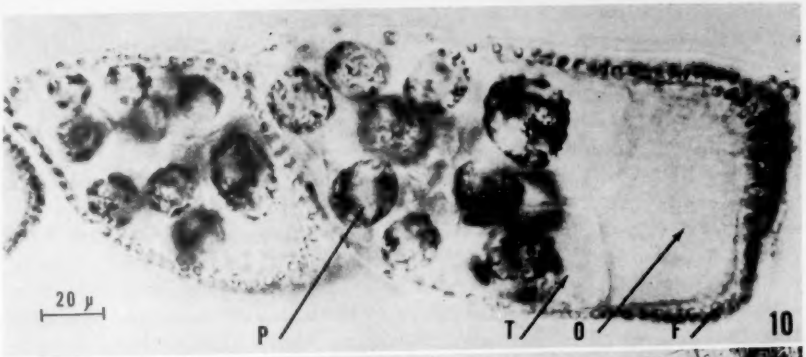
Genotype	Age in hours	Number of preparations	Appearance
sn^1/sn^1	156 ± 12	27	Abnormal
sn^2/sn^2	180 ± 12	20	Normal
sn^3/sn^3	120 ± 12	26	Normal
sn^4/sn^4	144 ± 5	13	Normal
sn^5/sn^5	156 ± 12	9	Abnormal
sn^{34e}/sn^{34e}	120 ± 24	18	Normal
sn^{50k}/sn^{50k}	156 ± 12	14	Abnormal
sn^{36a}/sn^{36a}	varying*	219	Abnormal
sn^3/sn^{36a}	168 ± 10	20	Normal
sn^2/sn^{36a}	156 ± 12	8	Normal
sn^{34e}/sn^{36a}	168 ± 24	13	Normal
sn^4/sn^{36a}	156 ± 12	15	Normal
sn^{36a}/sn^2sn^2	156 ± 12	2	Abnormal
sn^{36a}/sn^1sn^3	156 ± 12	2	Abnormal
sn^{36a}/sn^1	156 ± 12	1	Abnormal
sn^1sn^2/sn^1sn^2	156 ± 12	14	Abnormal
sn^1sn^3/sn^1sn^3	156 ± 12	10	Abnormal
sn^1sn^4/sn^1sn^4	156 ± 12	12	Abnormal
sn^2sn^3/sn^2sn^3	156 ± 12	13	Abnormal

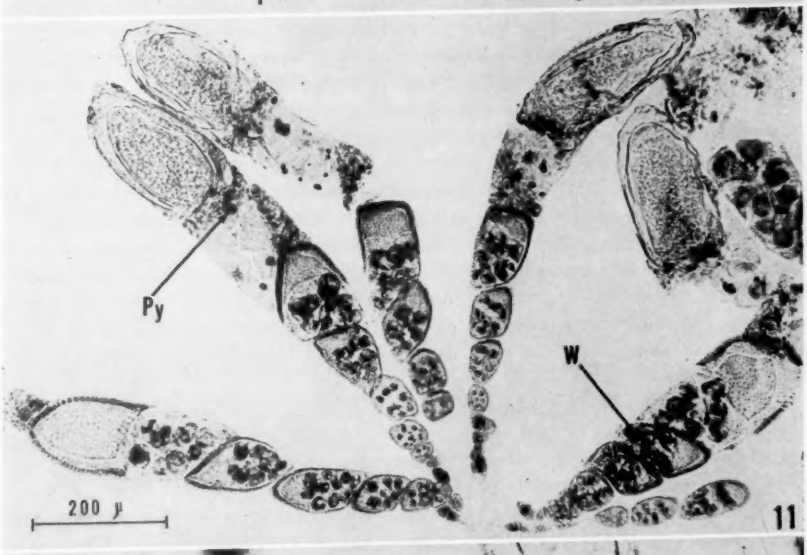
	Appearance	Number
Totals	Normal	122
	Abnormal	104
	Abnormal (sn^{36a})	219
		445

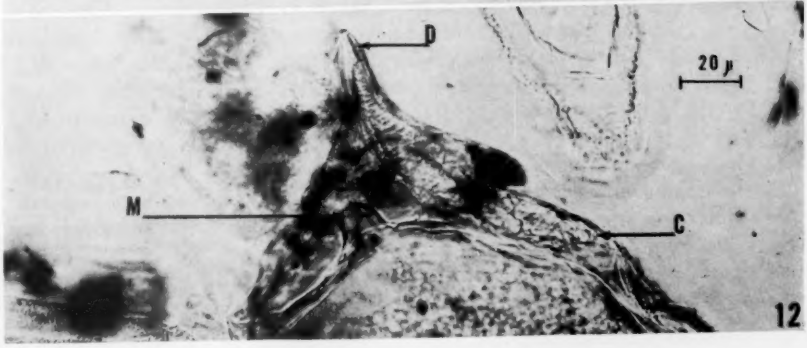
* See Table 6.

into subcategories IIa and IIb. Class IIa is represented only by the sn^{36a} mutant. KING and BURNETT (1957) reported several abnormalities of the 168-hour ovarian tissue in sn^{36a} flies. These observations have been verified and supplemented (BENDER and KING 1958) and show that nurse cell nuclei of the chambers past stage 4 contain abnormally large plasmosomes. (It is possible that the total amount of plasmosomal material remains unchanged, but that one large plasmosome is formed instead of several smaller ones). Further the Feulgen-positive material is abnormally distributed and reduced in quantity (Figure 10). Fusion

FIGURES 10–12.—Photographs of Feulgen-stained whole mounts of ovarian tissue. FIGURE 10.—Stage 7 and 9 follicles of a 168-hour sn^{36a} mutant. Notice the peripheral distribution of Feulgen-positive material and large plasmosome (P) in the trophocyte nucleus. A columnar follicular epithelium (F) surrounds the oocyte (O). The cytoplasm of a large, posteriorly located trophocyte (T) in the stage 9 follicle is indicated. FIGURE 11.—Several ovarioles of a 168-hour sn^{36a} mutant. Many of the chambers are wider than long (W). Pycnotic nurse cell nuclei (Py) are apparent. FIGURE 12.—A stage 13 chamber from a 156-hour sn^1 ovary demonstrating the blunted micropylar cone (M), shortened dorsal filament (D), and chorion (C). Note the vacuolated and nonhomogeneous appearance of the yolk.


20 μ
P
T
O
F
10


Py
W
200 μ
11


D
20 μ
M
C
12

of adjacent chambers in 168-hour sn^{36a} ovarian tissue occurs rarely, but the incidence of fusion increases with age. The volumes of the nuclei of the nurse cells are markedly increased in the mutant chambers. Chambers corresponding in volume to stage 6 and 7 contain nuclei equivalent in volume to those characteristic of stages 8–10. Oogenesis apparently ceases at stage 13. This is a slightly arbitrary statement, since the distinction between stage 13 and stage 14 in wild type flies is largely based on different stages in the development of the chorion, micropylar apparatus, and dorsal appendages. In *sn* mutants the development of these structures is so abnormal that they are no longer suitable for identifying stages.

Examination of the ovarian tissue of the sterile categories of *sn* mutants demonstrates that the method of classification of the chambers developed by KING, RUBINSON and SMITH (1956) cannot be applied readily to stages 11 through 14. Increase in the volume of yolk is inhibited to a greater extent than is formation of the dorsal appendages. Consequently, dorsal appendages which normally appear at stage 13 are present at a stage which would be classified as 11 with respect to volume of yolk. This observation demonstrates the rather autonomous behavior of the dorsal appendages which develop at the appropriate time independently of the volume of ooplasm.

Ovaries of 96- to 168-hour flies appear quite similar. Several ovarioles of a 168-hour sn^{36a} mutant are shown in Figure 11. Many chambers are rectangular with the long axis perpendicular to the long axis of the ovariole, whereas the wild type chamber is elongated in the direction of the axis of the ovariole thereby confirming earlier observations of KING and BURNETT (1957). Pycnotic nurse cell nuclei are quite noticeable in degenerating chambers. Oogenesis has usually advanced no farther than stage 11. Degeneration of chambers often occurs before this stage is reached.

Class IIb contains all other sterile *sn* mutants studied. All members of this group show similar cytological abnormalities. Again, the shape of the developing chambers in optical section often is rectangular with the long axis perpendicular to the long axis of the ovariole. The younger chambers appear to have been subjected to compression. Such a compression would be expected if early stages in oogenesis proceed at a normal rate, whereas later stages are retarded. Fully developed eggs of normal appearance are never present. The most advanced stage attained was 13, but these eggs were markedly abnormal. Dorsal filaments (as in sn^{36a}) were twisted and malformed, appearing shortened and thickened (Figure 12). The micropylar cone, when visible, seemed shorter and wider than in wild type eggs (Figure 12). Perhaps abnormalities of the micropyle prevent fertilization. The chorion forms late and seldom completely surrounds the anterior regions of the developing oocyte. MOHR (1922) reported that sn^1/sn^1 females lay approximately the same number of eggs as heterozygous controls. He further noted that eggs of homozygous flies are short, blunt and wrinkled, and that their dorsal filaments are small and blunt and are inserted at a more central position than in eggs of wild type flies. MOHR's observations were corroborated by this study. In addition, eggs deposited by the sterile class were amorphous and thus differed strik-

ingly from the firm, plump, and well-formed eggs of fertile heterozygotes. Abnormalities of the chorion, at least in part, accounted for these differences. Yolk is often abnormally vacuolated in contrast to the homogeneous yolk of fertile heterozygotes. The occasional necrotic appearance of the yolk of the most developed chambers could result from their retention within the ovariole for abnormally long periods of time. Sterility in this class of mutants could well be a consequence of aberrant yolk and/or inability of sperm to penetrate the malformed micropyle.

Several interesting contrasts between oogenesis in class IIa and IIb can be made. Approximately two percent of the total chambers observed in the 144-hour sn^{36a} ovaries are degenerating as indicated by the presence of pycnotic nuclei. This represents more than a ninefold becrease from the number of degenerating chambers observed in class IIb ovarian tissue of similar age. As expected there is a significantly larger percentage of younger chambers in sn^{36a} ovaries.

Comparative studies of stage distribution between the two sterile categories and the wild type (Table 5) display several other interesting differences. Note that the sn^{36a} ovary has a higher percentage of chambers in all the stages of development (except the most mature chamber present, stage 13) than does the sn^{1} mutant. Further, the percentage of chambers in yolk-forming stages (8–13) is greater in the sn^{36a} ovary than the sn^{1} ovary (58% *vs.* 38%). Obviously vitellogenesis is retarded in the sn^{36a} ovary. A retardation would account also for the fact that the modal yolk stage of the IIa group is 13 while that of the IIb group is 11. This retardation of vitellogenesis is markedly shown in the IIa group by

TABLE 5

Stage distribution in sn^{36a}, sn^{1} *and wild type ovarian tissue of similar age*

		sn^{36a} 144±6 hours (II a)		sn^{1} 156±4 hours (II b)		+ type 168 hours	
		n=27		n=27		n=10*	
	Stage	Percent	$\pm(s_{\bar{x}})\%$	Percent	$\pm(s_{\bar{x}})\%$	Percent	$\pm(s_{\bar{x}})\%$
No yolk	1	9.11	0.04	8.18	0.06	13.59	0.83
	2	10.79	0.04	6.60	0.06	11.12	1.32
	3	9.90	0.03	1.99	0.03	10.13	1.10
	4	10.52	0.04	8.81	0.07	6.49	0.00
	5	0.80	0.01	0.21	0.01	3.19	0.55
	6	10.70	0.04	8.28	0.05	13.10	3.36
	7	10.34	0.04	7.44	0.06	12.88	1.54
Yolk forming	8	9.28	0.04	8.28	0.07	6.60	0.88
	9	4.16	0.02	1.36	0.02	3.96	0.38
	10	3.45	0.03	1.05	0.01	4.62	0.93
	11	16.00	0.05	2.41	0.02	0.44	0.22
Yolk formed	12	1.41	0.02	0.73	0.01	0.66	0.33
	13	3.54	0.09	44.65	1.22	0.22	0.22
	14	....	...	....	...	12.99	1.76
Total percent		99.99		100.00		99.99	

* King (1957).

comparisons with IIb where the volume of yolky ooplasm is calculated in the two groups; whereas the volume of yolky ooplasm per female is $14 \times 10^7\ \mu^3$ in *sn*^{36a} females (both stocks have 15–16 ovarioles per ovary). Chamber volumes are calculated from the formula $v = 0.5236\ w^2\ l$, where w = maximum width and l = maximum length (KING, RUBINSON and SMITH 1956).

It would thus seem that the abnormalities displayed by the two classes of mutants are closely related, differing mainly in the degree. The one possible exception is the abnormal behavior of the nurse cell nuclei of the IIa group. However, it is possible that this too will turn out in the course of further investigations to be a more drastic reflection of a similar effect. Cytochemical studies with the light microscope of sections of the yolk of these two groups and among members of the IIb category could be expected to provide further contrasts.

Stage distribution in sn^{36a} *as a function of time:* A study was made of the stage distribution of the developing chambers in ovaries of *sn*^{36a} homozygotes of varying ages. These data are summarized in Table 6 and compared with information for wild type ovaries of various ages in Figure 13. It appears that development can proceed to stage 13 by 144 hours, and that by 432 hours the majority of chambers have reached this developmental stage where their maturation is impaired. The percentage of chambers in stage 5 is considerably reduced in comparison with wild type flies, whereas stages 8 and 11 are significantly higher than in the latter. The high percentage of chambers in stage 13 of the 432-hour flies is reflected by the tandem arrangement of two or more of these follicles per ovariole.

TABLE 6

Stage distribution as a function of age of sn^{36a} *ovarian tissue*

		A		B		C		D		E	
		12 hrs.		42±18 hrs.		96±6 hrs.		144±6 hrs.		432±6 hrs.	
	Stage*	Percent	±($s_{\bar{x}}$)%	Percent	±($s_{\bar{x}}$)%	Percent	±($s_{\bar{x}}$)%	Percent	±($s_{\bar{x}}$)%	Percent	±($s_{\bar{x}}$)%
No yolk	1	15.38	2.85	11.32	0.16	9.30	0.99	9.11	0.04	6.83	0.06
	2	15.38	2.85	11.01	0.06	10.85	0.97	10.79	0.04	9.02	0.06
	3	15.38	2.85	13.21	0.17	12.40	1.05	9.90	0.03	9.02	0.07
	4	3.08	0.51	12.58	0.14	10.08	0.92	10.52	0.04	9.88	0.07
	5	1.54	0.51	0.51	0.03	0.78	0.19	0.80	0.01	0.37	0.01
	6	33.85	5.96	12.26	0.16	9.30	0.84	10.70	0.04	10.61	0.08
	7	15.38	2.24	11.64	0.12	11.63	0.87	10.34	0.04	12.68	0.08
Yolk forming	8	....	...	11.32	0.24	15.50	0.89	9.28	0.03	5.37	0.04
	9	....	...	7.55	0.02	6.20	0.55	4.16	0.02	2.20	0.04
	10	....	...	4.72	0.09	2.33	0.19	3.45	0.03	0.60	0.01
	11	....	...	1.26	0.06	11.63	0.67	16.00	0.05	0.98	0.02
Yolk formed	12	....	...	0.94	0.03	....	...	1.41	0.02	....	...
	13	....	...	1.57	0.06	....	...	3.54	0.09	32.44	0.22
	14	....	...	....	...	....	...	....	...	....	...
Total percent		99.99	...	100.01	...	100.00	...	100.00	...	100.00	...
Number of examined ovaries			6		17		4		27		21

* Stage as percent of total number of chambers.

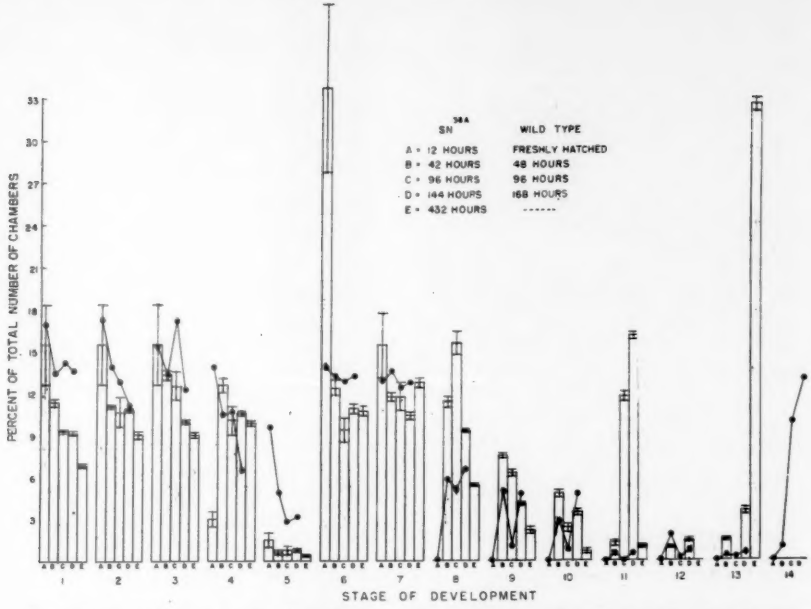


FIGURE 13.—A histogram showing the percent of the total number of sn^{36a} chambers in any stage of development in ovaries from flies of different ages. Comparisons may be drawn with information for wild type ovaries of various ages superimposed on the histograph in line-graph form.

The most advanced oocyte in each ovariole was recorded, and on the basis of these data it is possible to select the modal oldest stage (underlined in Table 7). The modal oldest stages are plotted in Figure 14 and are compared with similar information for wild type flies presented by KING and WOLFSBERG (1957). It is evident that oocyte development in the sn^{36a} homozygote lags considerably behind that of the wild type, and that oogenesis is never brought to completion (stage 14). No stage 14 chambers were observed in more than 100 sn^{36a}/sn^{36a} ovaries of varying ages.

Comparative studies of stage distribution as a function of age on the IIa group and wild type indicate that the rate of oogenesis is decreased, especially in the yolk-forming stages. This finding is further substantiated when the amount of yolk/fly, calculated for the class IIa category for flies of various ages, is compared with similar information for wild type flies (Figure 15).

Examination of Figure 15 shows that the amount of yolk in the ovaries of group IIa flies of advancing age generally remains lower than that of wild type flies of identical age. The total amount of yolk produced by the wild type and sn^{36a} females differs even more significantly, however, since wild type females under optimal conditions lay daily 20 times as many eggs (60 *vs.* 3) as do sn^{36a} females.

TABLE 7

Stage distribution as a function of age of sn^{36a} *ovarian tissue*

	Stage*	A 12 hrs.	A $\pm(s_{\bar{x}})\%$	B 42 hrs.	B $\pm(s_{\bar{x}})\%$	C 96 hrs.	C $\pm(s_{\bar{x}})\%$	D 144 hrs.	D $\pm(s_{\bar{x}})\%$	E 432 hrs.	E $\pm(s_{\bar{x}})\%$
No yolk	1	....	...	....	...	....	...	....	...	....	...
	2	....	...	....	...	....	...	....	...	0.60	0.01
	3	19.51	5.50	1.77	0.08	2.63	0.66	0.90	0.01	1.21	0.06
	4	2.44	8.09	3.54	0.13	5.26	1.31	0.30	0.01	2.41	0.02
	5	....	...	1.77	0.02	....	...	0.30	0.01	....	...
	6	53.66	9.39	7.08	0.28	....	...	3.89	0.02	2.41	0.12
	7	24.40	3.54	12.39	0.25	2.63	0.66	4.79	0.06	9.04	0.18
Yolk forming	8	....	...	26.55	0.65	26.32	1.68	11.68	0.06	8.43	0.68
	9	....	...	20.35	0.27	18.42	2.01	7.49	0.04	5.42	0.05
	10	....	...	15.04	0.28	7.90	0.66	7.19	0.05	1.81	0.15
	11	....	...	3.54	0.51	36.84	1.68	46.41	0.32	2.41	0.54
Yolk formed	12	....	...	3.54	0.09	....	...	3.89	0.02	....	...
	13	....	...	4.42	0.19	....	...	13.17	0.30	66.27	0.34
	14	....	...	....	...	....	...	....	...	....	...
Total		100.01		99.99		100.00		100.01		100.01	
Number of examined ovaries		6		17		4		27		21	

* Stage as percent of total number of the oldest (most posterior) chambers in the ovariole.

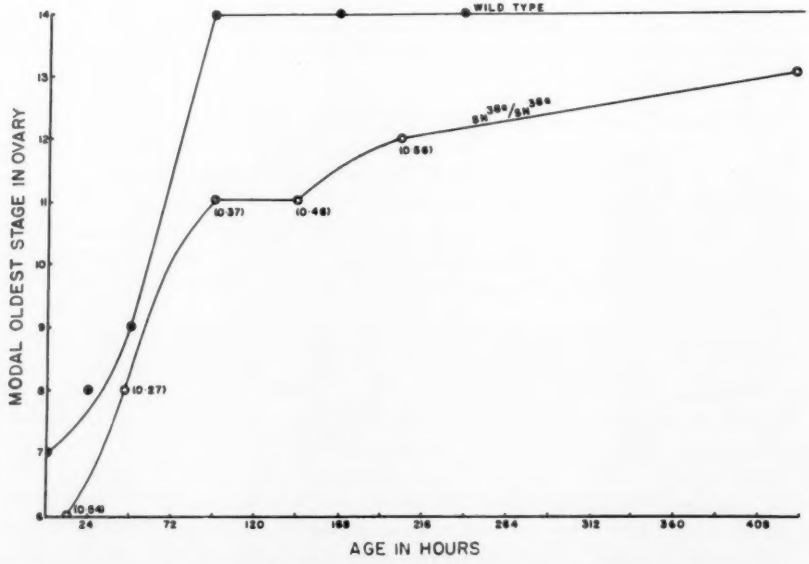


FIGURE 14.—A graph of modal oldest stage in ovary plotted as a function of age in hours.

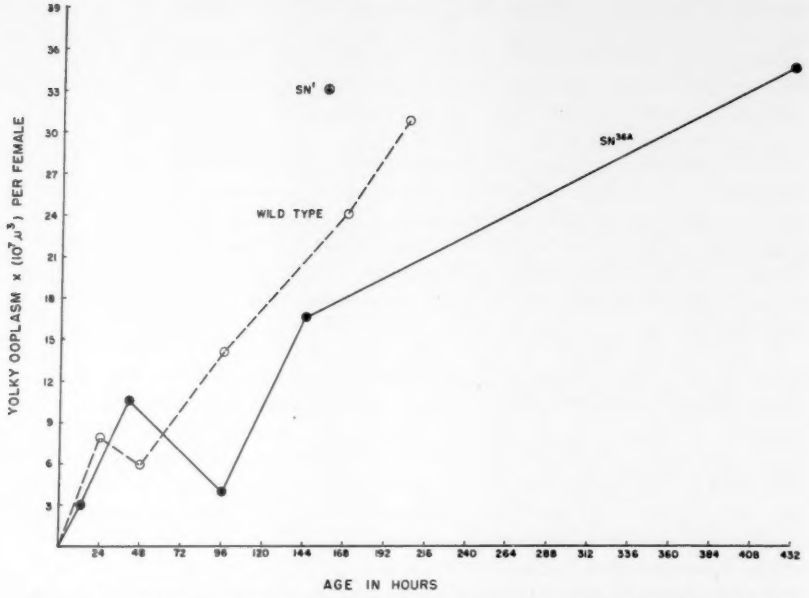


FIGURE 15.—Volume of yolky ooplasm × ($10^7\mu^3$) per female plotted as a function of age for *sn*^{36a} and wild type flies. (Data for 1-, 2-, 4-, and 7-day wild type flies from KING [1957]).

Also the *sn* stocks have 20 percent more ovarioles (31 *vs.* 25), thus the wild type data should be adjusted upwards an apportional amount to render the groups comparable. (There were generally seven chambers/ovariole in the 144- to 168-hour *sn*^{1}, *sn*^{36a} and wild type stocks).

The major particulate constituents of the insect yolk, protein and lipid yolk bodies, are probably of trophocyte origin in Drosophila (HSU 1953; KING and DEVINE 1958; KING and BURNETT 1958). In light of this evidence it seems reasonable to suppose that the trophocytes of the sterile singed mutants are unable to carry out their normal role in vitellogenesis. The abnormal appearance of the nurse cell nuclei in *sn*^{36a} mutants strengthens this assumption.

To summarize, cytological evidence suggests that vitellogenesis is retarded in ovaries of the sterile singed mutants, whereas preyolk chambers develop normally. Vitellogenesis appears most drastically retarded in *sn*^{36a}. Studies of the stage distribution of developing chambers in *sn*^{1} and *sn*^{36a} flies and comparative values for the amount of yolky ooplasm produced by these two groups once again indicate that vitellogenesis is more drastically affected in the *sn*^{36a} group. Studies of stage distribution as a function of age in the *sn*^{36a} group and in wild type flies showed that the rate of vitellogenesis in the *sn*^{36a} group is markedly decreased and that oogenesis is never brought to completion.

DISCUSSION

Since the *sn* mutants constitute a pseudoallelic series, it is of value to examine the available information concerning the phenotypic effects and spatial configurations of this series with the view of developing an hypothesis which could explain the action of this complex upon fertility and upon setal morphology. HEXTER (1957) divided the mutants into three phenotypic groups on the basis of data on bristle-hair morphology and fertility. Each group of mutants was considered to occupy a specific locus. The present study suggests, instead, the existence of four phenotypic groups on the basis of setal morphology since the sn^{36a} mutant appears to stand apart from the others. Further, three different phenotypic categories can be distinguished on the basis of cytological examination of ovarian tissue and progeny tests.

A chromosomal model can be developed which takes into consideration all pertinent information currently available for the *sn* complex. This model is illustrated by Figure 16. The model incorporates three main loci within the *sn* complex. All three loci are assumed to affect setal morphology (b of Figure 16) by the production of an unknown factor (substance y); only the first and third loci affect fertility by production of another factor (substance x). These products may or may not be related. The presence of a singed mutant either partially or completely inactivates the locus in question.

The locus proximal to the centromere is occupied by mutants sn^{1} and sn^{50k}. It is suggested that this locus is actually composed of two subloci (b and f) possibly separable by crossing over (BURDETTE 1949). The middle locus is occupied by mutants sn^{2} and sn^{4}. The locus farthest from the centromere is occupied by the sn^{3} and sn^{36a} mutants and resembles the first locus in that it is composed of two subloci (b and f), probably separable by crossing over. It is hypothesized that the sn^{36a} mutant causes greater inactivation (perhaps even complete inactivation) of the fertility factor than the sn^{3} mutant and an incomplete inactivation of the bristle-hair factor; whereas the converse holds for the sn^{3} mutant.

Such a proposal can possibly be tested experimentally. Mutants of the sn^{36a} group can be subjected to irradiation in an attempt to further inactivate the setal factor. If mutants can be recovered showing the bristle-hair morphology of the class 3 group (sn^{3}) and having modified oogenesis similar to the class 4 group

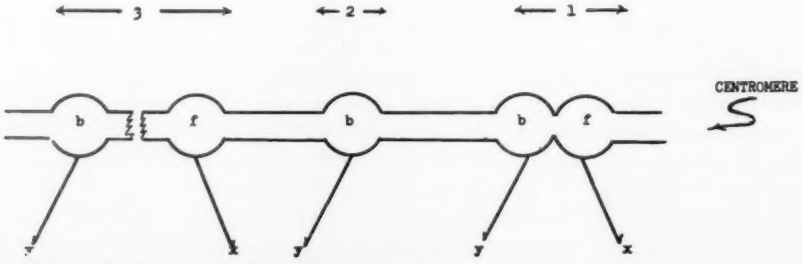
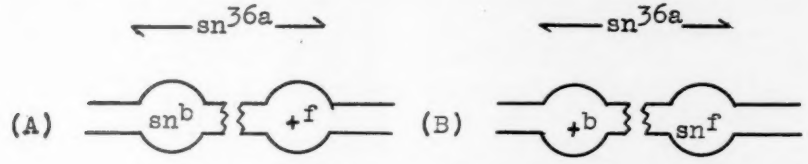


FIGURE 16.—A proposed model of the singed complex.

(sn^{36a}), this hypothesis will be strengthened. The reciprocal approach, namely irradiation of the sn^3 mutant and subsequent study of the ovarian tissue, is too laborious since this would necessitate investigation of the ovarian tissue of every female tested. By assuming the presence of a separable sublocus for fertility within the sn^3–sn^{36a} locus (Figure 16), it is not only possible to develop an explanation for the unexpected intermediate phenotypes observed when sn^4 and sn^{36a} mutants were examined in crossover experiments (HEXTER, unpublished), but to establish the relative positions for the setal and fertility moieties. In recombination experiments, expected wild type individuals from sn^{36a}–sn^4 crossovers were not completely wild type, and the presumed double mutant sn^{36a}–sn^4 was more nearly wild type than singed. Such a finding is what one might expect if the crossover occurred between the sn^{36a} sublocus for setal morphology and the sublocus for fertility (Figure 17) and if the resulting chromosomal segment of (A) pro-



duces more of a singed effect than the resulting chromosomal segment of (B). Both segments of (A) and (B) are affected by the type of neighboring fertility factor since they are distinguishable from sn^3 and sn^{36a}. The two chromosomes resulting from recombination (A and B) could be tested for fertility. If the progeny have arisen as a result of crossover between the setal sublocus and the fertility sublocus as considered in Figure 17, then (A) the singed product should be fertile, whereas (B) the nearly wild type product should be highly infertile. However, if the fertility factor of the sn^3–sn^{36a} sublocus lies to the left, then a crossover between these factors could be expected to produce an infertile, more nearly singed product

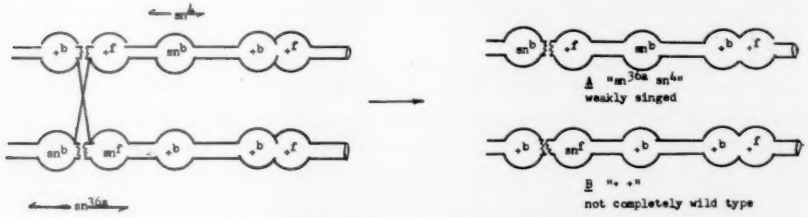


FIGURE 17.—Diagram of an hypothetical crossover between the fertility and the bristle-hair subloci of the sn^{36a} locus (developed from the model shown in Figure 16). The presence of a singed mutant is indicated by a "*sn*" in the appropriate position in the model with the bristle (b) or fertility (f) sublocus indicated by a superscript. Conversely, a wild type allele is indicated with a "+" sign. The products of such a crossover are indicated as "A" and "B". "A" is predicted to have mildly singed bristles and hairs and to be fertile; whereas "B" has only the fertility sublocus of the sn^{36a} locus and is expected to be nearly wild type but infertile.

with features of oogenesis comparable to those of the sn^{36a} group. In either case such findings would provide strong evidence of a separable fertility sublocus within the third main locus of the singed complex.

SUMMARY

1. Fourteen singed stocks were examined with respect to setal morphology and fertility. These stocks could be subdivided into four categories: (1) sterile, with kinky hairs and gnarled bristles—sn^1, sn^5, sn^{50k}, sn^1sn^2, sn^1sn^3, sn^1sn^4, sn^2sn^3, sn^{72}, and sn^c; (2) fertile, with kinky bristles only—sn^2, sn^4, and sn^{44e}; (3) fertile, with kinky hairs and gnarled bristles—sn^3; and (4) sterile, with gnarled bristles only —sn^{36a}

2. The phenotypic consequences of the genotypic combinations of the four *sn* classes were examined with respect to: (A) Fertility—any individual compounded with a fertile *sn* allele on one chromosome was fertile, and any individual homozygous for any two sterile *sn* alleles was sterile and (B) Setal morphology—Class 2 bristle and hair morphology is dominant over Class 3 and Class 4, and blends with Class 1. Class 4 bristle and hair morphology is dominant over Class 1 and 3.

3. Two major types could be recognized utilizing a study of oogenesis in the *sn* mutants. I. Normal oogenesis occurs in all fertile *sn* mutants. II. Abnormal oogenesis occurs in infertile mutants. The sterile category can be further subdivided into IIa and IIb with class IIa being represented only by the sn^{36a} mutant. Class IIb contains all of the sterile *sn* mutants studied except sn^{36a}. Cytological evidence suggests that vitellogenesis is retarded in ovaries of the sterile singed mutants; whereas preyolk chambers develop normally. Vitellogenesis appears more drastically retarded in the sn^{36a} group. Studies of the stage distribution of developing chambers in 156-hour sn^{36a} and sn^1 flies and comparative values for the volume of yolky ooplasm produced by these two groups once again indicate that vitellogenesis is more drastically affected in sn^{36a} flies. Studies of stage distribution as a function of age in sn^{36a} and in wild type flies showed that the rate of vitellogenesis in the sn^{36a} class is markedly decreased and that oogenesis is never brought to completion.

A model is described which attempts to explain the action of this complex locus upon fertility and upon bristle and hair morphology. This model contains three subunits, all portions of which deal with bristle-hair development. Two of the subunits are in turn divided into a bristle and fertility moiety. A separable fertility factor is hypothesized in connection with the subunit associated with the sn^3 and sn^{36a} mutants.

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LINKAGE AND FUNCTION IN NEUROSPORA[1]

S. R. GROSS[2] AND ADELE FEIN

Rockefeller Institute, New York, N.Y.

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TIGHT linkage of genes controlling the synthesis of enzymes involved in related physiological reactions is prevalent in Salmonella and *Escherichia coli* (DEMEREC and HARTMAN 1959). Extensive linkage of this sort has not been reported in Aspergillus and Neurospora. Instead, most of the genes concerned with the reaction sequences studied in the enteric bacteria have been found to be distributed throughout the genome of the fungi. Departures from a completely random distribution of functionally related genes have been reported frequently (see PONTECORVO 1958, for review). However, if one excludes those cases which probably involve complex cistronic relationships relative to the synthesis of a single protein, few linked loci have been observed that are functionally related but distinct. Moreover, tight linkage of more than two distinct functionally related genes has, up to now, not been established in the fungi.

The question can then be asked whether clustering of functionally related genes is a result of a selective mechanism peculiar to the physiological activity of the enteric bacteria or whether the selective mechanism is universally operative but more subtly expressed in other organisms. In this report evidence is presented indicating that the genetic determinants of four enzymes involved in the synthesis of the precursors of the aromatic amino acids and *p*-aminobenzoic acid (PAB) in Neurospora are closely linked. Furthermore, some specific qualitative and quantitative changes in the enzymatic complement of the mutant strains will be discussed.

Specific interest in the linkage relationship of the genes controlling the synthesis of the enzymes involved in the synthesis of the aromatic ring arose as a consequence of an apparent paradox displayed by a mutant strain of *Neurospora crassa* (C161), obtained by METZENBERG and MITCHELL (1958), which did not utilize shikimic acid for growth as a sole replacement of the requirement for the aromatic amino acids and PAB. The genetic and biochemical evidence suggested that this mutant was unable to carry out a reaction prior to the synthesis of the hydroaromatic compounds that have been established as intermediates in the synthesis of the aromatic amino acids of *E. coli* (DAVIS 1955) and Neurospora (TATUM, GROSS, EHRENSVÄRD, and GARNJOBST 1954). Failure of this mutant strain to respond to shikimic acid for growth as distinct from another, Y7655, (TATUM *et al.* 1954) which does, suggested that either an absolute permeability

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[2] Present address: Microbiology Dept., Duke University Medical Center, Durham, N.C.

barrier to shikimic acid was present in C161 or that the mutant strain was not capable of converting shikimic acid to the aromatic moiety of the amino acids as well as being blocked in a reaction prior to the synthesis of 5-dehydroquinic acid (DHQ), the first of the hydroaromatic precursors of shikimic acid (WEISS, DAVIS, and MINGIOLI 1953). The data presented below indicate that C161 is missing the entire enzymatic apparatus necessary to convert 2-keto-3-deoxy-D-*arabo*-heptonic acid 7-phosphate (KDHP) to at least one product of shikimic acid.

MATERIALS AND METHODS

Biological: Table 1 contains all pertinent information regarding the origin of mutant strains used in this investigation. The symbols *arg*, *arom*, *inos* and *pan* refer to gene loci that are involved in the synthesis of arginine, the aromatic amino acids and PAB, inositol, and pantothenic acid, respectively. Locus numbers were assigned to *arom* mutants in the order in which sufficient genetic and/or functional dissimilarities were observed. Since subsequent investigation has indicated that C161 probably is an aberration of some sort, *Ab* has been added to the locus designation (*arom-2*) assigned and the locus number reserved for the point mutation involving the first missing reaction in the metabolic sequence in strains bearing *arom-2 Ab*. The mutant strains will be referred to by their locus designation throughout.

The standard types used were 38-2a and 38-8A. These were obtained from SY7A and Y7655a (*arom-1*) after five successive sib crosses. Both *arom-2 Ab* and *arom-3* were backcrossed three times to the standard strains in order to obtain reasonable homogeneity and the proper heterocaryon compatibility background.

The growth requirements of the *arom* mutants were satisfied by the addition of 40 mg each of phenylalanine, tyrosine, tryptophan and 0.25 mg PAB per liter of VOGEL's synthetic medium (VOGEL 1956). Shikimic acid, 400 mg per liter was

TABLE 1

Mutant strains

Locus designation	Isolation number	Mutagen	Reference
arg-5	27947	X-ray	B*
arom-1	Y7655	N-Mustard	B*
arom-2 Ab	C161†	UV	METZENBERG and MITCHELL (1958)
arom-3	C163†	UV	METZENBERG and MITCHELL (1958)
bal: balloon	B56	UV	PERKINS (1960)
fl: fluffy	Lindegren	Spontaneous	B*
inos	89601†	N-Mustard	B*
pan-1	5531	X-ray	B*
pe: peach pe^{m}	Y8743m	Spontaneous?	B*

* B. BARRATT *et al.* (1954).
† Derivatives of the original isolates that would form heterocaryons with Stanford-Yale stocks were used.

added in place of the aromatic amino acids and PAB. The requirements for arginine and inositol were satisfied by the addition of 100 mg L-arginine and 10 mg of inositol per liter. Crosses of the *arom* mutants were found to be most fertile when the crossing medium was supplemented with 0.1 to 0.25 of the aromatic requirement. Random ascospore isolates were obtained by the sorbose plating method (NEWMEYER 1954).

Chemical: The strains used in the enzymatic analysis were grown in standing culture for three to four days in synthetic medium supplemented with the aromatic amino acids plus any other specific compound required for growth. The grinding and extraction procedure has been described (GROSS 1958). Generally, only a two- to four-hours extraction in 0.1 M Tris-HCl buffer *p*H 8.0 was employed.

In order to avoid error introduced by the conversion of DHS to PCA by 5-dehydroshikimic acid dehydrase (DHS dehydrase) and the endogenous TPN reduction exhibited by fresh extracts of Neurospora, measurements of the specific activity of 5-dehydroshikimic acid reductase (DHS reductase and 5-dehydroquinase) were done on 0 to 0.75 saturated $(NH_4)_2SO_4$ fractions after freezing and thawing (which inactivates more than 80 percent of the DHS dehydrase). 2-Keto-3-deoxy-D-*arabo*-heptonic acid 7-phosphate synthetase (KDHP synthetase) was measured in 0.4 to 0.55 $(NH_4)_2SO_4$ fractions dialyzed for 12 hours against M/30 *p*H 7.4 KPO_4 buffer with 10^{-4} M thioethanol. 5-Dehydroquinic acid synthetase (DHQ synthetase) was measured in similarly dialyzed 0.35 to 0.75 saturated $(NH_4)_2SO_4$ fractions. PCA oxidase and DHS dehydrase were measured directly in crude extracts.

In all cases where no enzyme activity was found, the entire crude extract as well as precipitates obtained by stepwise $(NH_4)_2SO_4$ fractionation (0.2 saturated $(NH_4)_2SO_4$ per step) were examined for activity.

DHS reductase was measured in a reaction mixture containing 1 μ mole shikimic acid, 10 μ moles triphosphopyridine nucleotide (TPN), 0.1 ml enzyme preparation in 2 cc 0.1 M Tris-HCl buffer *p*H 8.0. The initial reaction velocity was determined by the increase in optical density at 340 mμ (Σ_{340} TPNH $= 6.22 \times 10^3$) during the first five minutes of the reaction.

5-Dehydroquinase activity was measured by following the production of 5-dehydroshikimic acid (DHS) from DHQ (MITSUHASHI and DAVIS 1954). The reaction mixture contained 0.1 ml enzyme preparation plus 1 μ mole DHQ in 3 ml 0.1 M Tris-HCl *p*H 7.4. Because of end absorption of the crude $(NH_4)_2SO_4$ fraction, the reaction velocity was determined by measuring the increase in absorption at 240 mμ (DHS *p*H 7.4 $\Sigma_{240} = 9.7 \times 10^4$) instead of 234 m$\mu$.

The activity of PCA oxidase and DHS dehydrase were measured in 2 ml 0.1 M Tris-HCl *p*H 7.4 by the spectrophotometric and colorimetric procedures described previously (GROSS, GAFFORD, and TATUM 1956; GROSS 1958).

KDHP synthetase activity was measured by the procedure of SRINIVASAN and SPRINSON (1959). The synthesis of DHQ from KDHP was followed by measuring the disappearance of KDHP colorimetrically and the appearance of DHQ as determined by bioassay using an *arom* auxotroph of *Aerobacter aerogenes* A170-

143S1 (SRINIVASAN, KATAGIRI, and SPRINSON 1959). All bacterial strains mentioned in this paper were the generous gift of DR. B. D. DAVIS.

A unit of enzyme activity is defined as the amount of enzyme that converts 1 mμ mole substrate per minute under the assay conditions described. Protein concentrations were determined by the procedure of LOWRY, ROSEBROUGH, FARR, and RANDALL (1951).

DHS and DHQ were isolated from culture filtrates of mutant strains of *E. coli* by the procedures of SALAMON and DAVIS (1953) and WEISS, DAVIS, and MINGIOLI (1953). Cyclohexylammonium erythrose 4-phosphate dimethylacetal and the barium salt of 2-keto-3-deoxy-D-*arabo*-heptonic acid 7-phosphate (KDHP) were the generous gifts of DR. D. B. SPRINSON. Sodium erythrose-4-phosphate was prepared by the procedure of BALLOU, FISHER, and MCDONALD (1955). Barium KDHP was converted to the sodium salt before use. All other compounds used were obtained from commercial sources.

EXPERIMENTAL RESULTS

General physiological and genetic information regarding arom *mutants:* The mutant *arom-1* (Y7655) has been shown to be unable to convert DHS to shikimic acid (TATUM *et al.* 1954). It accumulates some DHS and large quantities of protocatechuic acid (PCA) in its growth medium. It will grow on shikimic acid as a sole replacement of its requirement for the aromatic amino acids and PAB. Its growth on shikimic acid, both in liquid culture and on agar, attains the rate of the standard strains only after an extensive lag period when sucrose or glucose is supplied as the carbon source. No lag in response to shikimic acid is observed when succinate or aspartate serve as the carbon source (MARY HURD, unpublished observations). This phenomenon closely resembles the glucose inhibition of the induced synthesis of β-galactosidase in *E. coli*, which has been interpreted as being the result of the inhibition of the synthesis of the permeability mechanism for the inducer (COHN 1956). The *arom-1* locus has been found to lie distal to *pe* (peach) and proximal to *fl* (fluffy) on linkage group II (GARNJOBST in BARRATT, NEWMEYER, PERKINS, and GARNJOBST 1954).

Strains bearing *arom-2 Ab* (C161) have been found not to accumulate any of the hydroaromatic precursors of the aromatic ring (METZENBERG and MITCHELL 1958). Taken at face value this implies that a reaction is blocked prior to the synthesis of DHQ. However, it was observed that shikimic acid does not replace the aromatic requirement of *arom-2 Ab*. We have confirmed this and have shown that it also does not respond to DHS or DHQ. Failure to respond to the presumed intermediates and the absence of accumulated compounds related to the hydroaromatic compounds suggested, among other possibilities, that a permeability barrier to hydroaromatic compounds exists in those strains with C (Cal. Tech) genetic background, and/or *arom-2 Ab* is a mutation that leads to the complete loss of the reaction sequence involved in the synthesis of the aromatic ring at least from a step prior to the synthesis of DHQ to a product of shikimic acid.

None of 400 segregants from three successive backcrosses of *arom-2 Ab* to the

standard strains grew on shikimic acid in place of the aromatic requirement. Hence, if a genetically controlled permeability barrier were involved, its determinant must be closely linked to *arom-2 Ab*.

The *arom-3* mutant (C163), unlike *arom-2 Ab* and *arom-1*, is comparatively "leaky" and has only a partial requirement for PAB. It does not respond to shikimic acid for growth. A careful examination of the products accumulated during growth using sensitive chromatographic and bioautographic procedures (for methods employed see TATUM *et al.* 1954) revealed the presence of shikimic acid, DHS, and large amounts of PCA. The accumulation of shikimic acid by *arom-3* apparently was missed by METZENBERG and MITCHELL (1958) because of the relatively small quantity accumulated and the relative insensitivity of the bioassay employed by them. The accumulation pattern observed conforms with that expected for a mutant blocked in a reaction subsequent to the production of shikimic acid.

The physiological behavior of the double mutant *arom-2 Ab arom-3* is identical with *arom-2 Ab*. The *arom-1 arom-3* double mutant secretes the same compounds as does *arom-1*, but like *arom-3*, fails to respond to shikimic acid. Since all attempted crosses of *arom-1* by *arom-2 Ab* have been sterile, the *arom-2 Ab arom-1* double mutant has not been synthesized.

Pairwise tests of functional complementation of the *arom* mutants employing the usual criterion of heterocaryotic growth on minimal medium revealed that combinations of *arom-1* plus *arom-3* and *arom-2 Ab* plus *arom-3* rapidly formed prototrophic heterocaryons while *arom-2 Ab* plus *arom-1* failed to complement each other. Combinations of *pan arom-1* plus *inos arom-2 Ab*, when tested on medium containing the full supplement of aromatic amino acids, grew successfully thus ruling out the possibility of the production of a specific inhibitor of growth in the *arom-2 Ab* plus *arom-1* heterocaryon.

The evidence thus far presented is consistent with the following conclusions: (1) At least one common functional process is impaired in *arom-1* and *arom-2 Ab*. (2) In addition to the disfunction held in common, *arom-2 Ab* is unable to synthesize DHQ and is also unable to convert shikimic acid to some intermediate subsequent to it in the synthesis of the aromatic ring. (3) The metabolic disfunction of *arom-3* involves a reaction subsequent to the synthesis of shikimic acid. This reaction can proceed in both *arom-1* and *arom-2 Ab*.

Genetic analysis of arom *mutants:* The apparent functional "allelism" of *arom-2 Ab* and *arom-1*, as determined by the analysis of complementation, suggested that these mutants should behave as alleles in the strict genetic sense. However, complete sterility of crosses of *arom-1* by *arom-2 Ab* prevented a direct genetic analysis. Instead, a comparative analysis of linkage to linkage group II markers was performed.

Segregants bearing *arom-2 Ab* are strongly selected against in most crosses. Furthermore, *arom-2 Ab* strains are morphologically distinct from the standard type—they are paler in color, and macroconidia production is frequently restricted to the top of the slant. Upon prolonged incubation, microconidia are often produced. Since the diagnosis of *pe*^{m} depends upon color, conidial distribution and

the production of microconidia, a large error is introduced in scoring random segregants from crosses of *arom-2 Ab* by *pe*^{m}. Problems of allele recovery and scoring were partly avoided by performing a tetrad analysis using the double mutant *pe*^{m} *fl* which produces microconidia exclusively (BARRATT and GARNJOBST 1949) and allows the unequivocal detection of exchanges in the *pe*^{m} *fl* interval by the production of segregants with the characteristic *fl* phenotype.

The results of crosses of *pe*^{m} *fl* by all of the *arom* mutants in addition to *arg-5* are presented in Table 2. Asci which yielded representatives of at least three of the four spore pairs are tabulated as complete tetrads. In view of the poor yield of complete tetrads in crosses involving *arom-2 Ab*, the data obtained from those asci yielding members of two spore pairs from the same half of the ascus are included and referred to as half tetrads. Some multiple exchanges cannot be observed in half tetrads; hence the estimates of interval distances are biased downwards to the extent of the half tetrad frequency.

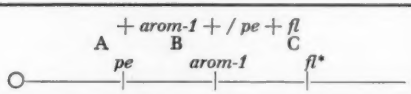
The data obtained are very heterogeneous with regard to interval lengths. This was expected in view of the heterogeneous origin of the mutants employed (STADLER 1956; MITCHELL 1959; PERKINS 1959). However, it can be concluded that: (1) All of the *arom* mutants are located in the right arm of linkage group II. (2) *arom-1* is apparently located between *pe* and *fl* (as observed by GARNJOBST in BARRATT *et al.* 1954). (3) The most probable position of *arom-3* is to the left of *pe* near *arg-5* and centromere.

The data from the *arom-2 Ab* by *pe*^{m} *fl* cross demonstrate an extreme shortening of the centromere to *pe* interval and the absence of exchanges between *pe* and *arom-2 Ab*. However, instead of a corresponding decrease in the *pe fl* region a slight increase in exchange frequency was observed.

The extreme contraction of the centromere to *pe* interval in the *arom-2 Ab*

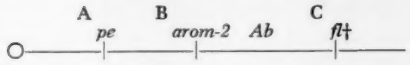
TABLE 2

Tetrad analysis of arom *and* arg-5 × pe fl *crosses*



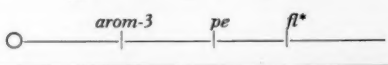
Tetrads isolated=124	Total exchanges			Complete tetrads=76 / Half tetrads=31 Multiple exchanges
Interval	A	B	C	1 A+B (2 or 4), 6A+C (2 or 4), 5A+C (3)
Complete tetrads	24	5	48	2 B+C (2), 2C+C (4),
Nonexchange tetrads = 19				2 A+C+C (2, 3 or 4 (A+C) 4C)
Half tetrads	10	2	7	None
Nonexchange half tetrads = 12				
Total	34	7	55	
Apparent interval length	16.0	3.3	26.0	

+ arom-2 Ab + / pe + fl



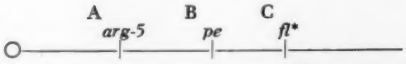
Tetrads isolated = 209			Complete tetrads = 61	Half tetrads = 57
Complete tetrads	1	0	42	1 A+C (2 or 4), 3C+C (4)
Nonexchange tetrads = 22				
Half tetrads	6	0	41	2 A+C (2 or 4), 2C+C (4)
Nonexchange half tetrads = 14				
Total	7	0	83	
Apparent interval length	3.0	0	35.0	

arom-3 + + / + pe fl



Tetrads isolated = 107			Complete tetrads = 94	Half tetrads = 12
Complete tetrads	12	34	53	3 A+C (3), 4 A+C (2 or 4), 7 B+C (3) 1 B+C (2), 1 B+C (4), 2C+C (4), 1 A+B+C (2 or 4(A+B) 2(B+C)), 1 A+B+C (2 or 4 (A+B) 4(B+C))
Nonexchange tetrads = 17				
Half tetrads	0	4	8	1 B+C (2), 1 C+C (4)
Nonexchange half tetrads = 2				
Total	12	38	61	
Apparent interval length	5.5	18.0	29.0	

arg-5 + + / + pe fl



Tetrads isolated = 97			Complete tetrads = 96	Half tetrads = 1
Complete tetrads	1	17	39	4 B+C (3), 1 B+C (2), 1 B+C (4), 2 C+C (4)
Nonexchange tetrads = 47				
Half tetrads	0	0	0	None
Nonexchange half tetrads = 1				
Total	1	17	39	
Apparent interval length	0.52	8.9	20.0	

* Gene order is derived from the data.
† Assumed order of *arom-2 Ab* and *pe*.
Numbers in brackets refer to the number of strands involved in multiple exchanges.

cross was accompanied by the appearance of a high frequency of spore abortion patterns (McCLINTOCK 1945) and poor germination among the normal eight spored asci. The fact that six out of seven of the exchanges in the centromere to *pe* interval were recovered in half tetrads might be indicative of selection against tetrads with exchanges in this region. However, an analysis of strand recovery among the incomplete tetrads failed to reveal any indication of preferential recovery different from that observed in the complete and half tetrads.

The apparent tight linkage of *arom-2 Ab* to centromere was also unexpected since an analysis of random segregants and tetrads from crosses of *arom-2 Ab* by *arg-5* and *arom-3* indicated that *arom-2 Ab* segregated fairly frequently from both loci (ten to 20 percent). The lengths of the *arg-5–arom-2 Ab* and *arg-5–arom-1* intervals were therefore compared by tetrad analysis. In the crosses listed in Table 3, the double mutants, *arg-5 arom-2 Ab* and *arg-5 arom-1* were crossed to

TABLE 3

Tetrad analysis of arom arg-5 *crosses*

arg-5 arom-1 / + +

Tetrads isolated = 120	Exchanges A	Exchanges B	Complete tetrads = 114 / Half tetrads = 4 Multiple exchanges
Interval			1 A + B (2 or 4), 1 A + B (3),
Complete tetrads	5	31	1 B + B (4)
Nonexchange tetrads = 81			
Half tetrads	0	2	1 B + B(4)
Nonexchange half tetrads = 3			
Total	5	33	
Apparent interval length	2.1	14.0	

arg-5 arom-2 Ab/ + +

Tetrads isolated = 120			Complete tetrads = 73 / Half tetrads = 26
Complete tetrads	2	28	1 A + B (3), 1 B + B (4)
Nonexchange tetrads = 45			
Half tetrads	1	1	none
Nonexchange half tetrads = 24			
Total	3	29	
Apparent interval length	1.5	14.7	

* Gene order is derived from the data.
Numbers in brackets refer to the number of strands involved in multiple exchanges.

the standard + + in order to maximize tetrad recovery. In this cross, as in all of those done with *arom-2 Ab*, many abortive asci were observed. However, the tetrad recovery from completely mature asci was greatly improved. The poor yield of *arg-5 arom-2 Ab* segregants, however, led to the recovery of a large proportion of the nonexchange tetrads as half tetrads. Exchanges in half and complete tetrads are therefore summed in order to avoid bias.

The data obtained for the *arg-5–arom-1* and *arg-5–arom-2 Ab* intervals are surprisingly homogeneous. Although variations of interval lengths observed in other crosses make determinations of confidence limits almost meaningless, the conclusion that *arom-2 Ab* and *arom-1* are at least closely linked is inescapable.

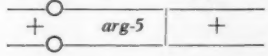
The tetrad analysis of the *arom-3* by *pe*^{m} *fl* cross indicated that *arom-3* was about 18 units proximal from *pe* in the region of *arg-5*. Although the linkage of *arom-3* to *arom-1* and *arom-2 Ab* is loose, in view of the paucity of information with regard to the relation of physical distance with "distance" in the recombinational sense, it seemed of interest to determine whether *arg-5*, a locus controlling an overtly different biochemical function, is located between the loci controlling aromatic biosynthesis.

The orientation of the *arg-5* and *arom-3* loci was determined with the aid of the colonial mutant—balloon (*bal*). PERKINS (1959) showed that *bal* was very close to the centromere of linkage group II and unequivocally established the order *bal – arg-5 – pe*. We have observed *bal* to segregate from centromere independently of *arom-3* (one out of 24 tetrads) which indicates that it is probably in the left arm of linkage group II.

TABLE 4

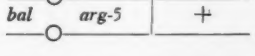
Three point crosses involving bal, arg-5 *and* arom-3

bal + *arom-3* / + *arg-5* +



Supplemented plates			Minimal plates–prototrophs				Interval length
Total viable spores*	Morphology *bal*	+	Total	Morphology *bal*	+	Frequency	*arg-5/arom-3*
8,060	3,660	4,400	22	22	0	0.00273	0.55

bal arg-5 + / + + *arom-3*



Supplemented plates			Minimal plates–prototrophs				Interval length
Total viable spores*	Morphology *bal*	+	Total	Morphology *bal*	+	Frequency	*arg-5/arom-3*
14,700	6,180	8,480	61	3	58	0.00415	0.83

* The viability of the spores plated was about 90 percent in both crosses.

Table 4 contains the results of an analysis of random segregants from crosses of *bal arg-5* by *arom-3* and *bal arom-3* by *arg-5*. Since the great majority of prototrophic segregants from the *bal arg-5* by *arom-3* crosses were morphologically + and from the *bal arom-3* by *arg-5* cross were *bal*, the order *bal–arg-5–arom-3* is established. The orientation of the *arom* mutants is such that no known mutant genes yielding auxotrophic phenotypes lie between *arom-1* (and *arom-2 Ab*) and *arom-3*. However, two loci controlling some morphological properties of Neurospora, *pe* and 584 (PERKINS, personal communication), are apparently located within the *arom-3* — *arom-1* interval.

Reversion of arom *mutants:* A careful examination of the relative reversion frequencies of the *arom* mutants was undertaken using *arom inos* and *arom pan-1* double mutants. Macroconidia were irradiated with doses of ultraviolet light leading to a survival of ten to 50 percent. Complete and partial reversions were selected for on medium supplemented only with calcium pantothenate and inositol and on medium supplemented with the above and PAB plus any two of the three required aromatic amino acids. The reversion frequency of *arom-1* was found to be highest when reversions for the tryptophan requirement were selected (7.5×10^{-5} per nucleus—assuming an average of two nuclei per macroconidium, and 6×10^{-6} per nucleus when selection for complete reversion was employed). Three out of four "reversions" of *arom-1* were found to be due to nonlinked suppressors. *arom-3* shows little variation in reversion frequency on the different selective media and has a mean reversion frequency of 2.5×10^{-7} per nucleus. No reversions of *arom-2 Ab* have been observed under comparable conditions among 5×10^{9} irradiated nuclei. Limitations of the number of conidia that can be plated without interfering with the growth of revertants (GRIGG 1952) prevent a more extensive quantitative search for reversions. However, massive transfers of *arom-2 Ab* conidia to selective media have never been observed to yield revertants under conditions in which contamination could be ruled out.

Enzymatic complements of the arom *mutants:* The growth response and accumulation pattern of the *arom* mutants suggested that *arom-1* is deficient in the ability to convert DHS to shikimic acid and that *arom-3* is unable to carry out some reaction subsequent to the synthesis of shikimic acid. The behavior of *arom-2 Ab*, however, was suggestive of a multiple loss of enzymatic functions in the synthesis of shikimic acid and one of its products. These conclusions were confirmed largely by the direct assay of the specific enzymes present in crude and partially purified extracts of the mutant strains.

The specific activities of 5-dehydroquinase, DHS reductase and PCA oxidase observed in extracts of the *arom* strains are listed in Table 5. These enzymes could be assayed directly with little error. The specific activities of KDHP synthetase and DHQ synthetase could not be determined accurately because of the presence of dephosphorylating enzymes and the apparent involvement of some unanalysed side reaction of DHQ or DHS. However, the assay procedures are sensitive enough to detect at least ten percent of the activity observed in enzyme preparations obtained from the standard strains (the sensitivity was determined in mixtures of extracts of *arom-2 Ab* and the standard strains). The determina-

TABLE 5

Enzyme complements of arom *and standard strains*

Enzyme	*arom-1*	*arom-2 Ab*	*arom-3*	Standard
KDHP synthetase	+	+	+	+
DHQ synthetase	+	0	+	+
5-dehydroquinase	0.80	0	2.73	0.77
DHS reductase	0	0	28.7	10.6
PCA oxidase				
-not induced	2.4	<0.01	2.5	<0.01
-induced*		0.62		0.36
DHS dehydrase	+	0	+	0

Enzymatic activity is expressed as units of activity per mg protein in fractions described in MATERIALS AND METHODS. Each value is the average specific activity observed in at least two independent preparations. +—Enzymatic activity was observed, but the determination of specific activity is subject to error—see text. 0—No activity observed. *—The synthesis of PCA oxidase was induced by the addition of 100 mg vanillic acid per liter growth medium.

tion of the specific activity of DHS dehydrase is subject to considerable error because of the instability of the enzyme. However, the sensitivity of the assay permits the detection of at least five percent of the minimum activity observed in *arom-1* and *arom-3*. The observed specific activity of DHS dehydrase varied between 0.1 to 0.5 of that found for PCA oxidase. Because of the errors in the determination of the specific activities of DHS dehydrase, KDHP synthetase and DHQ synthetase, only the presence or absence of detectable quantities of these enzymes in the various preparations is indicated in Table 5.

It should be pointed out that the enzymatic conversion of KDHP to DHQ is a rather complex reaction (SRINIVASAN *et al.* 1959). The participation of only one enzyme in the reaction has as yet not been completely established (SRINIVASAN, personal communication). For the sake of simplicity we refer to the enzyme or enzymes as DHQ synthetase.

The data in Table 5 confirm most of the deductions drawn from the information previously presented. DHS reductase is absent in crude extracts of both *arom-1* and *arom-2 Ab*. In addition, *arom-2 Ab* is apparently missing the entire enzymatic apparatus for converting KDHP to DHS while retaining the ability to synthesize KDHP from erythrose-4-phosphate and phosphoenol pyruvate. The entire sequence of reactions studied is present in *arom-3*, thus supporting the notion that *arom-3* is blocked in a reaction subsequent to the synthesis of shikimic acid. The specific activity of dehydroquinase and DHS reductase were found to be considerably higher in *arom-3* than in *arom-1* or the standard strains. However, the probable lack of isogenicity between the strains obfuscates any special significance of these observations.

The enzymatic reactions subsequent to shikimic acid have not as yet been elucidated. However, much can be deduced from the states of induction of DHS dehydrase and PCA oxidase in the various mutants. It has been shown that PCA is derived directly by dehydration of DHS (GROSS 1958). This reaction is irreversible and greatly favored during growth of mutants blocked subsequent to the synthesis of DHS thus leading to the accumulation of PCA as the major metabolic

product (some mutants blocked in the synthesis of phenylalanine and tyrosine have also been found to accumulate PCA). PCA is a substrate and an inducer of the synthesis of PCA oxidase (GROSS and TATUM 1955; GROSS 1959). Extracts of *arom-1* and *arom-3* contain PCA oxidase at the earliest stages of mycelial growth. The accumulation of PCA, the presence of DHS dehydrase and PCA oxidase are then excellent criteria for the involvement of a metabolic block subsequent to the synthesis of DHS. The virtual absence of these two enzymes in extracts of *arom-2 Ab* therefore seems to be a consequence of its inability to synthesize the hydroaromatic compounds. The induction of PCA oxidase synthesis in *arom-2 Ab* is equivalent to that observed in the standard type when vanillic acid or PCA (inducers of PCA oxidase) are added to the growth medium (GROSS and TATUM 1955).

The reactions involved in the synthesis of shikimic acid and the aromatic amino acids are summarized in Figure 1. 5-Phosphoshikimic acid is included in the scheme as a probable intermediate. This compound is accumulated by many *arom* mutants of *E. coli* (DAVIS 1955) and has been implicated in the synthesis of anthranilic acid (SRINIVASAN 1959). Although it has not as yet been demonstrated that 5-phosphoshikimic is derived from shikimic acid by direct phosphorylation, it is provisionally considered to be the immediate product of shikimic acid. Phosphorylated intermediates are usually not accumulated in culture filtrates of Neurospora, presumably because of the presence of active phosphatases (AMES and MITCHELL 1955). Compound "?" is included in the scheme as an hypothetical intermediate to explain the genetic and biochemical information described below.

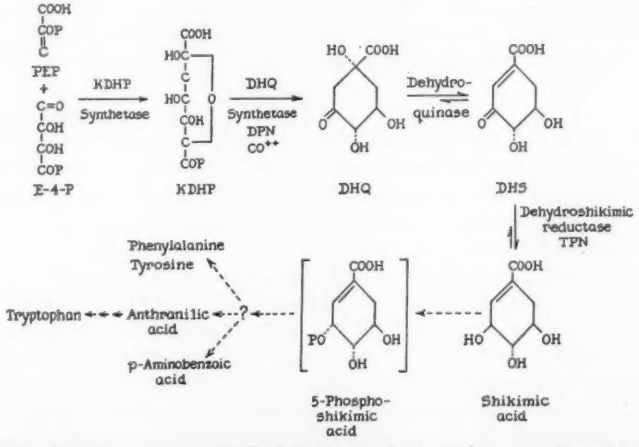


FIGURE 1.—Reaction sequence involved in the synthesis of the aromatic amino acids and PAB. Brackets signify that the compound has not been definitely established as an intermediate. PEP, phosphoenolpyruvate; E-4-P, erythrose 4-phosphate; KDHP 2-keto-3-deoxy-D-*arabo*-heptonic acid 7-phosphate; DHQ, 5-dehydroquinic acid; DHS, 5-dehydroshikimic acid; TPN, triphosphopyridine nucleotide.

The isolation of additional arom *mutants:* Although *arom-2 Ab* is phenotypically similar to *arom-1* in its inability to synthesize DHS reductase and is closely linked to *arom-1*, the infertility of crosses between these mutants has prevented the direct demonstration of allelism. The coincidental deficiency of DHS reductase in both mutants and the deficiency in the synthesis of at least two additional enzymes in *arom-2 Ab* strongly suggest that the individual genetic determinants of the synthesis of the deficient enzymes are included within the *arom-2 Ab* region. Support for this conclusion could be obtained by the examination of the linkage relationships of mutants controlling reactions deficient in *arom-2 Ab* other than the conversion of DHS to shikimic acid.

In addition to the above, it was clear that additional support was required for the conclusion that *arom-2 Ab* does not respond to shikimic acid for growth as a consequence of its inability to carry out a reaction subsequent to the synthesis of shikimic acid. As indicated previously, the inability of *arom-2 Ab* to utilize shikimic acid for growth in place of the aromatic supplement could conceivably be due to the loss of a permeability mechanism. Proof of the deficiency in *arom-2 Ab* of a reaction subsequent to shikimic acid synthesis could be obtained if a mutant were found which failed to respond to shikimic acid, did not complement *arom-2 Ab*, but did complement *arom-1* and *arom-3* and, in addition, was tightly linked to *arom-1*.

A search for *arom* mutants was therefore undertaken employing the "inositol-less death" procedure (LESTER and GROSS 1959). After permitting the inactivation of UV irradiated conidia to proceed for four days on minimal sorbose medium or medium supplemented with phenylalanine, tryptophan and PAB, the plates were supplemented with inositol plus either the entire growth factor requirement of the *arom* mutants or tyrosine. Shikimic acid proved to be a poor supplement for the selection of *arom* mutants, for the growth of *arom-1* on shikimic acid in sorbose medium is very slow.

Fourteen *arom* mutants as well as a large number of unclassified auxotrophs were obtained from three experiments. The *arom* mutants fell into two classes. The first of these consisted of 11 mutants which behaved as heteroalleles of *arom-3*. Their behavior, in complementation tests, suggested the existence of five functional subareas of the *arom-3* locus. One of the mutants of this class—R2212, which forms a slow growing prototrophic heterocaryon with *arom-3*, has been examined genetically as well as enzymologically. It falls in the same region of linkage group II (closely linked to *arg-5*) as does *arom-3*, rarely forms prototrophic segregants in crosses with *arom-3* and is biochemically indistinguishable from *arom-3*.

The other group obtained consisted of three mutants which were of the desired phenotype. They did not complement *arom-2 Ab*, but, unlike *arom-2 Ab*, did complement *arom-1* as well as all members of the *arom-3* class. These mutants did not respond to shikimic acid as the sole replacement of their aromatic growth requirement. Shikimic acid, DHS and PCA were secreted during growth, and mycelial extracts contained the full complement of enzymes necessary for the production of shikimic acid from its precursors. These mutants are therefore blocked

in a reaction subsequent to the production of shikimic acid. Their failure to complement *arom-2 Ab* indicates that *arom-2 Ab*, in addition to its deficiency of the three enzymes necessary to convert KDHP to shikimic acid is also deficient in the ability to convert shikimic acid into one of its products. Thus, it is not necessary to invoke the notion of a permeability barrier to explain the failure of *arom-2 Ab* to grow on shikimic acid.

The search for *arom* mutants was by no means exhaustive and, in fact, was terminated upon obtaining the desired class of mutants. DR. R. W. COLBURN (personal communication) obtained a number of *arom* mutants by a modification of the selection procedure of WOODWARD, DEZEEUW, and SRB (1954) after X-ray treatment. Four of these were found to be alleles of *arom-1*.

Genetic analysis of arom-4 *mutants:* The locus designation *arom-4* was assigned to the new class of *arom* mutants, and one of them, R2204, (referred to hereafter as *arom-4*) was used in the genetic analysis.

An analysis of random segregants from crosses of *arom-4* by *arg-5 pe*^{m} *fl* and *arom-3* revealed that *arom-4* is within the same area of linkage group II as is *arom-1*—approximately four units from *pe* between *pe* and *fl* and about 20 units from *arg-5*. The direct determination of allelism of *arom-4* with *arom-2 Ab* could not be done because crosses of the two, like crosses of *arom-1* by *arom-2 Ab*, have been completely sterile. However, a critical estimate of the linkage of *arom-4* to *arom-1* and the order of the loci with respect to *arg-5* was obtained from the three point cross—*arg-5 arom-1* by *arom-4*—summarized in Table 6.

Since there was no obvious selection against the recovery of *arg-5* segregants, and all of the segregants prototrophic with respect to the aromatic requirement did not require arginine for growth, the order *arg-5 – arom-1 – arom-4* is established.

TABLE 6

Three point cross–arg-5 arom-1 + / + + arom-4

	arg-5	*arom-1*	+*
	+	+	*arom-4*
Spores plated on arginine only†			11,700
Prototrophs for aromatic requirement			19
Genotype of prototrophs	*arg-5* + +		0
	+ + +		19
Frequency of recombinants			0.00162
Apparent interval length			0.32
Spores plated on arginine and aromatics			835
Number of segregants isolated			200
Phenotypes‡	*arg arom*		107
	+ *arom*		92
	+ + (genotype = + + +)		1

* Gene order is derived from the data.
† Approximately 84 percent of the spores plated were viable.
‡ No distinction was made between *arom-1* and *arom-4*.

The interval between *arom-1* and *arom-4* was found to be quite small (0.32 units). If one assumes that the *arom-1* — *arom-4* interval measured is reasonably close to the average interval length for the postulated loci controlling the synthesis of the hydroaromatic intermediates, then an approximate length of one unit is obtained for the entire cluster.

The physiological deficiencies of the *arom* mutants are illustrated in Figure 2, and the linkage relationships are illustrated in Figure 3.

There is no direct evidence that the reaction controlled by *arom-4* precedes that of *arom-3* in the biosynthetic sequence. However, the association of the determinants of the three prior reactions in the sequence with *arom-4* within the *arom-2 Ab* aberration is prejudicial in favor of the order illustrated. Compound "?" is postulated as a common intermediate for all of the aromatic compounds as the simplest explanation for the quadruple requirement of *arom-3*.

DISCUSSION

Linkage and function: The data presented above indicate that the genes determining the synthesis of at least four enzymes involved in the sequential production of precursors of the aromatic amino acids are clustered in a rather small region of linkage group II of Neurospora. This constitutes the first demonstration of *extensive* linkage of the genetic determinants of sequential biosynthetic reactions in an organism other than *E. coli* and Salmonella. Gene clustering of this sort can therefore no longer be considered a result of a selective mechanism peculiar to the mode of physiological activity of the enteric bacteria. However, since most of the linked sequences studied in the enteric bacteria have been found to be quite randomly distributed in the genome of the fungi, serious restrictions

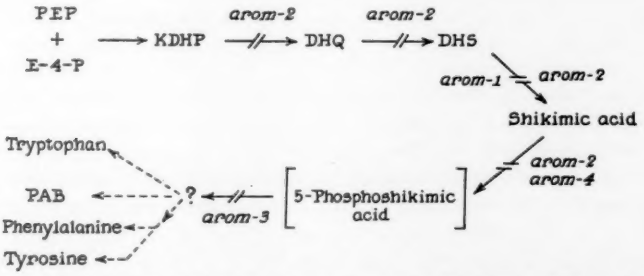


FIGURE 2.—Physiological deficiencies of the *arom* mutants. See legend of Figure 1 for definitions of abbreviations.

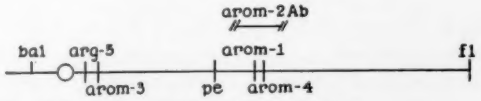


FIGURE 3.—Most probable linkage relationship of *arom* loci.

must be placed on general hypotheses concerning the maintenance of linkage in the two groups of organisms.

There appears to be no selective advantage for the concerted heterocatalytic functions of the linked genes in terms of the ultimate synthesis of essential metabolites. Heterocaryons of *arom-1* and *arom-4* are indistinguishable from the standard strains with regard to morphology and rate of growth. Furthermore, the significance of the relationship between linkage and the similarity of the compounds involved in the various enzymatic reactions is obscure. Each of the enzymes displays a different mechanism of catalysis and must, in addition to different binding sites for the various cofactors involved, have at least configurational differences about the substrate binding site. It was, therefore, no surprise to find that the separation of 5-dehydroquinase from DHS reductase could be almost completely accomplished simply by ammonium sulfate fractionation and/or ion exchange chromatography on diethylaminoethylcellulose (GROSS, unpublished).

JACOB (1959) has proposed that the rates of the heterocatalytic syntheses of a gene cluster are controlled at the chromosomal level by "repressers" (VOGEL 1957) which may, in some cases at least, be synthesized as a consequence of the specific reaction sequence involved. According to this view no *a priori* restrictions are placed on the nature of the reactions involved. They may be related in terms of a specific biosynthetic sequence as well as participation in a common physiological process. Thus, the genes determining λ coli-phage synthesis in *E. coli* K-12 are linked and are subject to repression (JACOB 1959) in a manner similar to that of the gene cluster determining the biosynthesis of tryptophan (COHEN and JACOB 1959). The selective advantage for clustering, then, would be derived not from the efficiency that might be obtained from an "assembly line" production of substrates but from the control of reactions by the alteration in the relative rates of synthesis of specific proteins during growth. According to this view, no intuitive restrictions are placed on the most advantageous arrangements of genes for any particular organism, and it becomes just as easy to envision the linkage of genes determining morphogenetic properties (e.g., *T*, *Ki*, *Fu* in the mouse; DUNN 1956) as the linkage of those involved in the production of essential metabolites. Hence, the different linkage pattern in the enteric bacteria and the fungi might not represent a tendency toward randomness in the fungi but, more interestingly from a physiological and developmental point of view, the requirement for different patterns of control of growth and differentiation in the two groups of organisms.

Repression of the synthesis of enzymes involved in related reactions by the products of the reactions has been primarily observed in *E. coli* and Salmonella (VOGEL 1957; PARDEE 1959; AMES and GARRY 1959). Although the activity of DHS dehydrase was found to be considerably higher in *arom-3* than in the standard strain, the variations observed could be due to genotypic effects other than repression. Repeated attempts to demonstrate repression of the enzymes of shikimic acid synthesis with the standard and mutant strains of Neurospora have failed. However, as AMES and GARRY (1959) have suggested, this might be a consequence of the inability at present to control the growth of Neurospora in such a way that it is not always maximally repressed.

The autoinduced biosynthesis of enzymes: That at least some enzymes in Neurospora are subject to wide variations in synthetic rate is obvious from the data presented with regard to PCA oxidase and DHS dehydase. The standard strain and *arom-2 Ab* produce very little of these enzymes during growth in the usual media. The synthesis of PCA oxidase, at least, can be induced in both of these strains by the addition of PCA or a number of analogues to the growth medium (GROSS and TATUM 1955). Of particular interest here is the fact that both DHS dehydrase and PCA oxidase are present in high concentration in *arom-1*, *arom-3* and *arom-4* as part of the constitutive complement of enzymes. Thus, the presence of these enzymes in the mutant strains apparently stems from the accumulation of their inducer-substrates as a consequence of interference in the normal biosynthesis of the aromatic ring. This enzymatic pleiotropy leads to the diversion of the precursors of aromatic biosynthesis into a constitutive series of reactions leading ultimately to their total catabolism (GROSS 1959).

Thus, whether or not one wishes to view enzymatic induction as a reversal of "repression" (VOGEL 1957), the heterocatalytic activity of genes of Neurospora are subject to wide variation. It is important to note in this respect that although DHS is an intermediate in aromatic biosynthesis the presence of DHS dehydrase could only be demonstrated under condition of DHS accumulation. Apparently the concentration of DHS in mycelia of the standard strains never reaches inducing concentrations.

Some aspects of arom-2 Ab: Failure of *arom-2 Ab* to complement *arom-1* and *arom-4* in heterocaryons apparently rules out the possibility that the enzymatic deficiencies observed in *arom-2 Ab* result from a primary block in the synthesis of DHQ, which is, in turn, required to induce the synthesis of the enzymes involved in subsequent reactions in aromatic biosynthesis. Furthermore, the abnormal segregation of *arom-2 Ab* and its failure to revert suggest that the genetic determinants of the enzymes responsible for the conversion of DHQ to a product of shikimic acid are involved in some relatively small chromosomal aberration.

Except for its anomalous segregation from *pe*, the segregation of *arom-2 Ab* from all linkage group II markers was found to be approximately the same as that of *arom-1*. In view of this and the apparent independent segregation of *arom-2 Ab* from linkage group I, IV and V markers (GROSS, unpublished observations), it does not seem likely that *arom-2 Ab* is an *extensive* inversion or translocation. It is possible that the spore abortion patterns observed in crosses *arom-2 Ab* did not result from chromosomal missegregation (MCCLINTOCK 1945) but instead, as a consequence of a deficiency in the spore maturation process. The lack of exchanges between *arom-2 Ab* and *pe*, as well as the suppression of exchanges in the *pe* to centromere interval observed in the cross of pe^{m} *fl* by *arom-2 Ab*, could be explained on the assumption that each of the mutants involves a very short inversion or deletion. If this were true, serious pairing difficulties would arise only in crosses involving the heterologous aberrations.

Neither the failure to revert nor the absence of several enzymatic activities can be taken as sufficient evidence for the specific involvement of a deletion in *arom-2 Ab*. First of all, reversion frequencies per nucleus vary quite widely, and the methods of detection in Neurospora become exceedingly crude below fre-

quencies of less than 10^{-9}. Secondly, the assumption that the loss of a number of heterocatalytic functions can only be interpreted as a consequence of a deletion does not seem at all necessary. Until further genetic and cytogenetic work can be done with *arom-2 Ab*, it seems best to consider *arom-2 Ab* some sort of small chromosomal aberration in the immediate vicinity of *arom-1* and *arom-4*.

SUMMARY

The genetic and physiological properties of several *arom* mutant strains of Neurospora are described. The most likely interpretation of the evidence presented is that the genes responsible for the production of at least four enzymes involved in the synthesis of the aromatic ring are closely linked in linkage group II. Another gene involved in the same reaction sequence, while also on linkage group II, is loosely linked to the others. The inability of *arom-2 Ab* to carry out four reactions in the sequence appears to be due to an aberration in the *arom-1* and *arom-4* region of linkage group II.

The problem of linked genes controlling related physiological reactions has been discussed in terms of the involvement of common control mechanisms for protein synthesis.

Autoinduced enzyme synthesis is proposed as the mechanism leading to the appearance of two enzymes, as part of the constitutive enzymatic complement of *arom-1*, *arom-3* and *arom-4*, that are present, if at all, only in extremely low concentrations in *arom-2 Ab* and the standard strains.

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MATERNAL EFFECT TRANSFERRED BY INJECTION AND FURTHER ANALYSIS OF TEMPERATURE EFFECTIVE PERIOD FOR TUMOROUS HEAD IN DROSOPHILA MELANOGASTER[1]

ELDON J. GARDNER, J. HOWARD TURNER, AND WILLIAM D. BERSETH

Utah State University, Logan, Utah

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INITIAL experiments (GARDNER 1948) on the tumorous head phenotype demonstrated a difference in the results of reciprocal crosses. A maternal effect dependent on a substance elaborated in the presence of a homozygous sex-linked gene (*tu-1*) was postulated (GARDNER and WOOLF 1949) to account for this observation. Changes in temperature during the early stages of development were found to alter the effect (GARDNER and WOOLF 1950).

Extensive reciprocal crosses between tumorous head (*tu-h*) and "wild" laboratory stocks (GARDNER and STOTT 1951), laboratory stocks carrying mutants (GARDNER, STOTT and DEARDEN 1952), and flies collected from natural populations (GARDNER and GARDNER 1953) further demonstrated the maternal effect. From most crosses between *tu-h* females and males from other stocks about 30 percent of the first generation progeny showed the tumorous head phenotype compared with about one percent or less from the reciprocal cross between females from most other stocks and *tu-h* males. Variations from this pattern were observed in some crosses. Modifiers and different genetic backgrounds were postulated to account for the differences. Penetrance, expressivity, and relative viability were described in the inbred *tu-h* stock following three years of selection and inbreeding (GARDNER and RATTY 1952). Experiments involving two series of backcrosses (GARDNER 1959) indicated that no persistent cytoplasmic substance is involved and that a single, recessive, sex-linked gene (*tu-1*) controls the maternal effect. An autosomal semidominant gene (*tu-3*) is necessary for any expression of the trait. There is a resemblance between the behavior of tumorous head and that of CO_2 sensitivity for which a genoid was described by L'HERITIER (1951). There are also conspicuous differences. The genoid is an independent virus or plasmagene while two major nuclear genes are involved in the tumorous head complex. No cytoplasmic particle comparable with the genoid of L'HERITIER has been detected in tumorous head flies.

The present experiments were designed to test the hypothesis that a substance elaborated by *tu-h* females carrying the gene *tu-1* in homozygous condition is responsible for the maternal effect. Further analyses of the temperature effective period which is associated with the maternal effect are also included in this report.

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MATERIALS AND METHODS

For the injection experiments, *tu-h* females were etherized and washed in two baths of distilled water to remove adhering food particles, salts, and other substances from the body surface. The flies were then crushed and homogenized with ten times their weight of insect Ringer's solution (1000 ml H_2O, 7.5 gm NaCl, 0.35 gm KCl, 0.21 gm $CaCl_2$). Crude extracts were lightly centrifuged at 2,800 rpm to remove solid particles and cellular debris. The supernatant was injected into the lateral thorax of wild (Lausanne) virgin females with a Dutky Fest microinjector. Injected females were mated with *tu-h* males, and the progeny were classified and compared with controls.

The standard corn meal, molasses, agar medium with live yeast added was used for all experiments. Flies were maintained at 25°C unless otherwise noted. Percentages are given in the tables with accuracy to the nearest whole number. When reciprocal crosses are symbolized, the female parent is always written first.

The first experiment, carried out to define the temperature effective period more narrowly, was prepared by mass mating 50 *tu-h* females with 50 wild males in dacron laying cages (SIMMONS and GARDNER 1958) and collecting eggs laid during two-hour intervals. Seven successive batches of eggs were deposited on separate petri dishes. Cultures were maintained at 21°C and 45 percent relative humidity except during the four-hour intervals at 30°C. Controls were similar to the experimental cultures but were not subjected to heat treatment. The first petri dish was placed in the 30°C incubator during the first four hours after the eggs were laid, the second was subjected to the higher temperature during the interval from four to eight hours after egg deposition, and so on at four-hour intervals for 24 hours. After eclosion, the adult flies were killed by freezing and classified for abnormal cephalic growths.

For the next experiment, designed to determine whether longer periods at higher temperature are more effective than four-hour intervals, another series of cultures was obtained as described above for the first experiment. Six experimental cultures were treated in the same way but all six were placed in the 30°C incubator immediately after egg deposition and left for different periods of time. The first was left four hours, the second, eight, and so on at increases of four hours until the last group which remained for 24 hours. Controls were maintained at 21°C and 45 percent relative humidity.

When it was shown that the abnormal growths being investigated could be altered by temperature changes during four-hour intervals in the early developmental period, a comparative study was made of the metabolic rates of developing larvae from different cultures during the first 24 hours after egg deposition. One hundred eggs were collected from each of the following: *tu-h* stock, wild stock, *tu-h* × wild, and wild × *tu-h* (female parent is always written first). Oxygen uptake during the first 24 hours after egg deposition was determined with a Warburg apparatus. Readings were taken at 30-minute intervals.

THOMPSON and TENNANT (1953) found by alternating wet and dry exposures that Drosophila larvae have a higher respiration rate in saturated air

than in dry air. Since humidity effects are closely allied with temperature effects, these two environmental factors were controlled as well as possible during the first 24 hours of development. Tests were made at 20°C with 20, 45, 52, and 66 percent relative humidity and at 30°C with 21, 45, 53, and 68 percent relative humidity. Eggs were laid at the temperature and relative humidity indicated, parent flies were removed, and cultures were allowed to remain under the same environmental conditions for 24 hours. Cultures were then removed to another incubator maintained at 24°C and 40 percent relative humidity. After eclosion the adults were classified for abnormal growths.

The atmospheric relative humidity which was recorded did not represent the effective relative humidity during the larval stage. Eggs were laid on the surface of the medium where the relative humidity of the air could have an influence, but when the larvae emerged from the eggs they went immediately into the medium which was saturated with moisture. The larvae were probably not influenced by the air above the medium under the conditions of this experiment. All cultures were as nearly alike as possible, with respect to the water content of the medium.

EXPERIMENTAL RESULTS

The results of the first injection experiment are summarized in Table 1. Four wild (Lausanne) females, injected with extract from adult *tu-h* females and mated with *tu-h* males, produced a total of 425 progeny with 76 or 18 percent abnormal. From two control matings, which were similar except that the wild females were not injected, 276 progeny were produced with two (one female and one male) abnormal. Another control experiment was carried out in which Lausanne females were punctured with the injection needle, but no extract was released. Four (three females and one male) of the 207 progeny were abnormal. When insect Ringer's solution was injected into Lausanne females, ten (eight females and two males) of the 264 progeny were abnormal. Results from controls were within the range obtained from previous matings between Lausanne females and *tu-h* males. Lausanne apparently has a genetic background which may increase the percentage of abnormal flies from this type of mating to as much as

TABLE 1

F_1 progeny from crosses between wild (Lausanne) females, injected with extract from tu-h *females and crossed with* tu-h *males. Results from controls and reciprocal crosses are included*

Treatment†	Abn. females	+ females	Abn. males	+ males	Percent* abnormal
Wild injected × *tu-h*	34	179	42	170	18
Control (not injected)	1	125	1	149	1
Punctured	3	96	1	107	2
Ringer's injected	8	132	2	122	4
tu-h × Lausanne (not injected)	67	150	77	171	31

* Percentages given with accuracy to nearest whole number.
† Female parent is always written first.

three or four percent of abnormal flies compared with less than one percent for most wild stocks. The small percentages (less than one percent) of abnormal flies from wild females and *tu-h* males were explained (GARDNER and WOOLF 1949) on the basis of independent action of gene *tu-3*. From the reciprocal cross between *tu-h* females and wild (Lausanne) males, 144 of the 465 flies were abnormal. This result is comparable with previous large-scale matings between *tu-h* females and Lausanne males.

The extract injection experiments on Lausanne females were repeated under carefully controlled conditions. Results of the second experiment are summarized in Table 2. Nineteen percent of the progeny of the injected females were abnormal compared with three and four percent from the two controls.

Injection experiments similar to those with Lausanne were conducted with the Samarkand wild stock. Among the numerous stocks with which *tu-h* has been outcrossed, Samarkand crosses have shown the lowest expression of tumorous head. Apparently the genetic background in this stock reacts in a different way than that of Lausanne. Results of the experiments involving Samarkand are summarized in Table 3. Extract from *tu-h* females was injected into Samarkand females which were later mated with *tu-h* males. Four percent of the progeny were abnormal. No abnormal flies were obtained from the control cross between uninjected Samarkand females and *tu-h* males. Samarkand females injected with Ringer's solution produced no abnormal flies. Extract from larval *tu-h* flies injected into Samarkand females did not alter the results, but females injected with extract from *tu-h* eggs and mated with *tu-h* males produced three percent of

TABLE 2

Results from second experiment similar to that reported in Table 1 in which extract from tu-h *females was injected into wild (Lausanne) females which were crossed with* tu-h *males*

Treatment	Abn. females	+ females	Abn. males	+ males	Percent abnormal
Wild injected × *tu-h*	41	122	17	127	19
Control (not injected)	1	55	3	72	3
Ringer's injected	8	132	2	122	4

TABLE 3

F_1 progeny from crosses between wild (Samarkand) females, injected with extract from tu-h *females and crossed with* tu-h *males. Results from controls and reciprocal crosses are included*

Treatment	Abn. females	+ females	Abn. males	+ males	Percent abnormal
Wild injected × *tu-h*	9	247	8	212	4
Control (not injected)	0	1,041	0	988	0
Ringer's injected	0	206	0	178	0
tu-h larval extract injected	0	66	0	76	0
tu-h egg extract injected	4	90	2	85	3
Wild (Lausanne) extract injected	0	101	0	110	0
tu-h × Samarkand	16	146	2	176	5

abnormal flies. Extract from Lausanne wild females was injected into Samarkand females which were mated with *tu-h* males. No abnormal flies were produced. Five percent of abnormal flies came from the reciprocal cross between *tu-h* females and Samarkand males.

Results from the first experiment designed to test the temperature effective period are summarized in Table 4. The maternal effect could be greatly increased by changing the temperature from 21°C to 30°C at any of the four-hour intervals between the 8–12 and the 20–24-hour periods after the eggs were deposited. Cultures subjected to increased temperature during the first two intervals (0–4 and 4–8 hours after egg deposition) were not significantly different from the control maintained constantly at 21°C. Results of the next experiment in which the cultures were left at 30°C for different time periods are summarized in Table 5. These results were not significantly different from those of the first experiment (Table 4), indicating that a four-hour interval was sufficient for the full effect.

Results of the oxygen consumption experiment are summarized in Table 6 and shown graphically in Figure 1. Progeny of the two reciprocal crosses (*tu-h* × wild and wild × *tu-h*) show no significant difference in oxygen consumption. Neither

TABLE 4

F_1 progeny from crosses between tu-h *females and Lausanne males subjected to 30°C at four-hour intervals during first 24 hours after egg deposition*

Time period	No. eggs	Adults classified				Percent abnormal
		Abn. females	+ females	Abn. males	+ males	
0–4 hours	136	17	27	6	24	31
4–8 hours	123	9	14	13	24	37
8–12 hours	174	24	21	47	7	72
12–16 hours	128	30	4	22	13	75
16–20 hours	154	40	12	21	15	69
20–24 hours	123	18	12	14	8	62
Control	131	10	24	21	34	35

TABLE 5

F_1 progeny from crosses between tu-h *females and Lausanne males subjected to different time periods at 30°C, each interval beginning with time of mating*

Time period	No. eggs	Adults classified				Percent abnormal
		Abn. females	+ females	Abn. males	+ males	
0–4 hours	366	79	215	37	35	32
8 hours	286	64	159	39	24	36
12 hours	421	119	68	161	73	67
16 hours	309	145	55	85	24	74
20 hours	241	100	31	87	23	78
24 hours	263	123	24	64	52	71
Control	578	118	156	128	149	45

TABLE 6

Measurements of oxygen consumed by eggs and larvae of different stocks and crosses given in microliters per egg during four-hour intervals

Time period	Source of samples			
	(1) *tu-h* × *tu-h*	(2) + × +	(3) *tu-h* × +	(4) + × *tu-h*
(a) 0–4 hours	22.824*	12.896	19.432	25.976
(b) 4–8 hours	18.512	39.496	43.432	47.952
(c) 8–12 hours	12.960	45.312	45.812	53.944
(d) 12–16 hours	46.656	96.712	102.872	115.872
(e) 16–20 hours	30.880	45.168	56.288	53.952
(f) 20–24 hours	31.720	49.208	55.144	58.984

* Oxygen consumption in microliters/per egg/four-hour period.

is there a significant difference between the results of the two reciprocal crosses and those of the wild stock. Analysis of variance showed the time period 12–16 hours (d at the left of Table 6) to be significantly different (one percent level) from c, e, and f. The differences c *vs.* e, f, and e *vs.* f were not significant. The cross 1 (*tu-h* × *tu-h*) sample was significantly different (one percent level) from 2, 3, and 4.

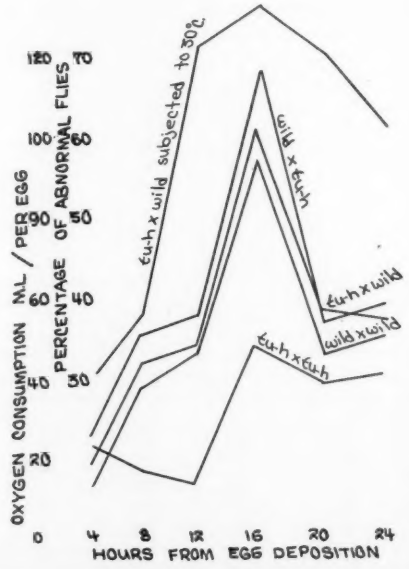
OXYGEN CONSUMPTION M.L. / PER EGG
PERCENTAGE OF ABNORMAL FLIES
HOURS FROM EGG DEPOSITION
tu-h x wild subjected to 30°C
wild x tu-h
tu-h x wild
wild x wild
tu-h x tu-h

FIGURE 1.—Oxygen consumption of samples of 100 eggs during first 24 hours after eggs were laid (data from Table 6). Upper curve represents the effect of increased temperature during first 24 hours after egg deposition (data from Table 4).

The graph at the top of Figure 1 represents the percentage of abnormal flies produced when cultures from the *tu-h* × wild cross were subjected to 30°C at four-hour intervals (from Table 4). There is a rough parallel between the rate of respiration and the temperature effective period for the tumorous head trait.

Under the conditions of these experiments, no significant differences in head abnormalities were related to humidity, but the relationships previously reported for temperature (GARDNER and WOOLF 1950) were confirmed. The results showed the present level of expression in the tumorous head stock after some 12 years of selection and inbreeding in our laboratory to be about 80 percent at 20°C and 90 percent at 30°C. They also confirmed the difference in reciprocal crosses, upon which the maternal effect hypothesis was based, with two wild stocks (Lausanne and Canton).

DISCUSSION

Transfer of the maternal effect from tumorous head females to wild females by injection of a crude extract supports the hypothesis (GARDNER and WOOLF 1949) that a tangible substance is involved. Previous experiments (GARDNER 1959) have shown that no persistent cytoplasmic factor is carried from generation to generation, but the possibility of a virus or a genoid (L'HERITIER 1951) acting under the control of a gene is not excluded.

The substance injected into the body cavity must be capable of penetrating the developing eggs in the ovary or tubes. KING and BURNETT (1959) have shown from autoradiographic studies that certain chemicals may be taken into the ovary of fruit flies. The *tu-h* substance depends on the sex-linked gene (*tu-1*), and its action is related to the action of the autosomal gene (*tu-3*) which must be present if any expression of the trait occurs in the adult flies. The substance may act by altering the dominance of the autosomal gene (*tu-3*). CAVALCANTI, LAGDEN, FALCAO, and CASTRO (1958) have described the interaction of a nuclear and cytoplasmic component in sex-ratio factor in *Drosophila prosaltans*, and MALOGOLOWKIN, POULSON, and WRIGHT (1959) have succeeded in transferring a maternally inherited sex-ratio in *D. willistoni*.

From the two injection experiments reported here, 18 and 19 percent of the progeny of injected females and *tu-h* males (simplex for *tu-1* and homozygous for *tu-3*) were abnormal. These results may be compared with 31 percent for the progeny of *tu-h* females (homozygous for *tu-1* and *tu-3*) and wild males. F_1 flies from both of these crosses would carry one *tu-3* gene; those from *tu-h* mothers showed the characteristic maternal effect, and those which were injected with *tu-h* maternal extract showed the same effect in a lesser but significant degree.

Tumorous head flies have now been crossed reciprocally with those of numerous laboratory stocks and wild populations. Most results have followed a fairly uniform pattern. About 30 percent of the progeny from the cross, *tu-h* females × wild males, were abnormal compared with one percent or less from the reciprocal cross, wild females × *tu-h* males. Some variations, presumably due to the genetic background in the stock to which outcrosses are made, have been obtained.

Stephenville females mated with *tu-h* males produced the highest proportion (89 percent) of abnormal flies (GARDNER and STOTT 1951). From the reciprocal cross between Stephenville males and *tu-h* females, 20 percent of abnormal flies were obtained. No abnormal flies have been obtained from the cross: Samarkand females × *tu-h* males, and only five percent of abnormal flies was obtained from the reciprocal cross. The genetic background in Samarkand is evidently better buffered against these abnormal expressions or more favorable for normal expression as compared with the genotypes of other stocks. Samarkand females injected with extract from *tu-h* females and mated with *tu-h* males produced four percent of abnormal flies. When an extract was prepared from eggs of *tu-h* females and injected into Samarkand females which were subsequently mated with *tu-h* males, three percent of the progeny was abnormal. Injection with *tu-h* larval extract had no effect; the results were similar to the uninjected controls in which no abnormal flies appeared.

Two approaches are being made to obtain data concerning the nature of the substance and the mechanics of the maternal effect. (1) Extracts are being refined and separated into their chemical components. Each component is being tested for transmission of the maternal effect. (2) The temperature effective period is being investigated more critically to see if some aspect of metabolic activity might suggest how the maternal effect operates.

To define the temperature effective period more narrowly than the 24-hour period detected in the previous study (GARDNER and WOOLF 1950), it was necessary to collect eggs rather than to transfer whole cultures to different temperature incubators. Laying cages were used to obtain large numbers of eggs from the same parents within a short time period. The results showed that any four-hour interval between 8–12 and 20–24 hours after the eggs were deposited was as effective as the full 24 hours at the higher temperature. The temperature effective period indicates that some chemical activity associated with the abnormality is in progress during this early period of development.

The over-all pattern of oxygen consumption conformed to that obtained by VAVALA (1954). In our experiments there was no significant difference between the results of the reciprocal crosses (*tu-h* × wild and wild × *tu-h*), and these results did not differ from those of the wild stock. The sample from *tu-h* consumed considerably less oxygen than the other three samples. This is probably because of the decreased viability of *tu-h* flies, particularly in the egg and larval stages (GARDNER and RATTY 1952). If there were fewer living larvae in the *tu-h* sample after the initial stage of the experiment compared with other samples, the amount of oxygen consumed would be considerably less than that of the other samples. Comparison between the graphs for the heat sensitive period of the *tu-h* × wild cross and the high oxygen consumption indicates that the heat sensitive period begins earlier and extends longer than the period of highest oxygen consumption.

SUMMARY

A crude extract, prepared from the bodies of tumorous head females, was injected into wild (Lausanne) females which were subsequently mated with tumorous head males. Eighteen and 19 percent of the progeny from two different

experiments expressed the tumorous head phenotype compared with one to four percent for the controls. This result supports the hypothesis that a tangible, transmissible substance is associated with the maternal effect. Similar injection experiments were conducted with the wild Samarkand stock. Four percent of the progeny from *tu-h* injected Samarkand females crossed with tumorous head males were abnormal compared with none for the uninjected controls. The temperature effective period during the first 24 hours of development was defined more narrowly. Four-hour intervals at 30°C between the 8–12 and 20–24-hour intervals after egg deposition were found to be as effective as longer periods at the higher temperature. Measurement of oxygen consumption during the first 24 hours of development showed a peak for the period 12–20 hours after egg deposition.

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THE EFFECT OF COPPER AND IRON SALTS AND TRYPTOPHAN ON HEAD ABNORMALITIES AND MELANOTIC TUMORS IN DIFFERENT STOCKS OF DROSOPHILA MELANOGASTER[1]

J. HOWARD TURNER AND ELDON J. GARDNER

Utah State University, Logan, Utah

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COPPER and iron compounds and tryptophan were added to the food medium and injected into larval and adult fruit flies, *Drosophila melanogaster*, in a series of experiments designed to test the effect of different chemicals on specific gene action. Salts of heavy metals are known to alter phenotypes (GOLDSCHMIDT and PITERNICK 1957; RAPOPORT 1939; SANG and MCDONALD 1954), and under certain conditions they act as mutagenic agents (HERSKOWITZ 1951; LAW 1938). Tryptophan was shown in a previous study (SIMMONS and GARDNER 1958) to be related to the proportion of flies with head abnormalities. Effects of copper and iron salts and tryptophan on the expression of head abnormalities, melanotic tumors, and general mortality of flies are reported.

MATERIALS AND METHODS

Two melanotic tumor stocks, tu^{50j} and tu^{150}, two stocks with structural abnormalities in the head region identified in previous reports as tumorous head stocks, *tu-h* and *w tu-h* (tumorous head with white eyes), and two wild inbred stocks, Canton and Stephenville, were used in the experiments.

The tu^{50j} stock was obtained in the summer of 1958 from W. J. BURDETTE at the University of Utah and maintained through inbreeding and selection in our laboratory. When the flies were kept at room temperature, between five and ten percent of the adults had melanotic tumors. The tu^{150} stocks had been inbred and selected for melanotic tumors over a period of ten years at Utah State University. While the experiments reported here were in progress, some seven to 14 percent of the adult flies had melanotic tumors which occurred most commonly in the abdomen but occasionally in the thorax. The tumors were colorless in early larvae and became pigmented as the larvae grew older. In the adults the tumors appeared as encapsulated masses of black pigment.

The *tu-h* phenotype and genetic mechanism have been described (GARDNER 1948; NEWBY 1949; GARDNER and WOOLF 1949; GARDNER and STOTT 1951; GARDNER and RATTY 1952). Some 86 percent of abnormal flies occurred in cultures maintained at 25°C during the period in which these experiments were in

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progress. In the *w tu-h* stock, the proportion of flies with head abnormalities was somewhat lower, ranging from 60 to 75 percent. Both wild stocks used in these experiments have been inbred for many years in our laboratory and are known to be free from major modifiers or alleles of the tumorous head genes. (A different Stephenville stock was found to give different results and was postulated (GARDNER and WOOLF 1949) to carry an allele of one of the tumorous head genes.)

The standard medium (corn meal, agar, molasses, with live yeast added) was used throughout the study. Aliquots of the experimental additives were introduced to measured quantities of hot medium and distributed in the medium by thorough stirring. Each chemical being tested was added to the live yeast suspension as well as to the basic medium. Pilot experiments were conducted to determine tolerances of the different Drosophila stocks to various concentrations of additives.

By making use of laying cages (SIMMONS and GARDNER 1958) through which parent flies could be transferred to a series of cultures for oviposition, the same individuals were used to produce eggs for an entire experiment. First instar larvae were collected by flooding (with water) the medium upon which the eggs were previously deposited. Ten larvae were inoculated into each of several six-inch test tubes containing slants of the medium appropriately prepared with additives for treatment. Tubes were maintained at 25° ± 1°C with a relative humidity of 45 ± 5 percent. Larvae reaching the pupal stage and pupae becoming adults were recorded, and percentages for treatments were calculated. Proportions of adults expressing melanotic tumors or head abnormalities were determined. Concentration levels of copper sulfate ($CuSO_4$) were 0, 160, 320, 640, 960, 1280, and 1600 μg/ml, and those of iron ammonium sulfate ($Fe(NH_4)(SO_4)_2$) were 0, 660, 1320, 1980, 2640, and 3300 μg/ml.

To check the results of the feeding experiments, chemicals were introduced directly into larvae and adults by injection. Some 25 larvae or 20 adult flies were anesthetized and placed at one time on a 1 × 3 inch microslide covered with scotch tape (sticky side up) for each operation. Slides were placed on the stage of a dissecting microscope, and chemicals were introduced with a microsyringe calibrated to deliver the same quantity in each injection. Operations were observed under a magnification of 150×. After injection the specimens were quickly removed from the slide, washed with Ringer's solution, allowed to recover from etherization in a moist chamber, and transferred to sterile dishes containing the standard medium. Mortality of larvae and adults was calculated and compared with that of the controls. Proportions of adults with abnormal expressions were tabulated.

EXPERIMENTAL RESULTS

Preliminary experiments with copper sulfate showed that concentrations of 160 μg/ml and 320 μg/ml were slightly toxic, 640 μg/ml was highly toxic, and no flies survived the 960 μg/ml level. A differential mortality occurred among the treated stocks; tumorous head flies being most susceptible. In these stocks the

controls with no additive showed a 60 to 70 percent mortality. At levels of 160 μg/ml and 320 μg/ml, 70 to 80 percent did not survive, and all flies died at the 640 μg/ml level. The melanotic tumor strain, *tu*^{150}, was intermediate, and *tu*^{50j} was quite similar to the wild Stephenville stock. Canton showed the lowest mortality at all treatment levels. Percentages of larvae, based on samples of 200, which failed to reach the adult stage are summarized in Figure 1. Results from the two *tu-h* stocks were not statistically different from each other but different from those of all others, whereas the two melanotic stocks were different from each other. Mortality occurred in both larval and pupal stages. In all cultures to which copper sulfate was added the developmental period was prolonged. A

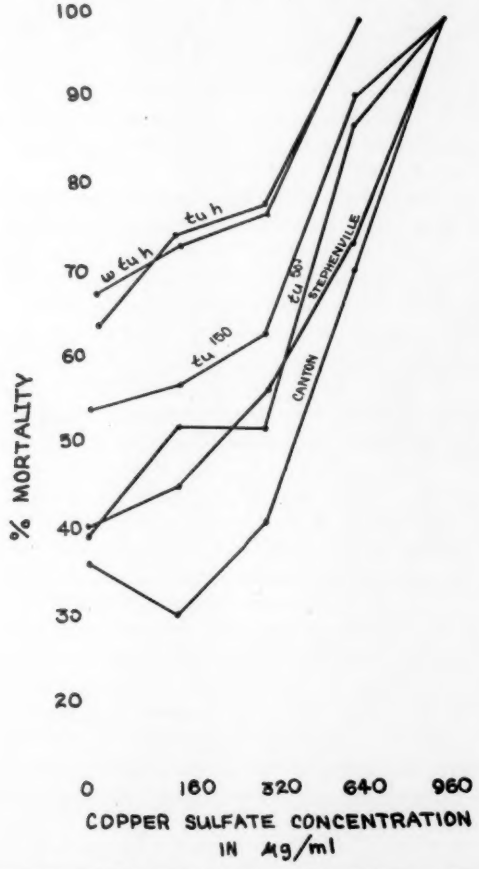

FIGURE 1.—Percentages of larvae which failed to reach adult stage when different levels of copper sulfate were added to food medium.

minimum of 14 days was required for the flies raised in a culture with excess copper to reach the adult stage compared with ten days for the controls. There was no statistically significant change in the expression of melanotic tumors or tumorous head expressions which could be attributed to the copper treatment.

A phenotypic change was observed when copper sulfate was added to the medium of the melanotic tumor strain, *tu*^{150}. In the culture to which 320 μg/ml of copper sulfate was added, 13 females and 12 males, from a total of 148 progeny, expressed eye abnormalities similar to some expressions of the mutant, eyeless. Among the 182 progeny from the culture with 160 μg/ml, one female and five males were eyeless. No such expression was observed among several thousand untreated flies of this stock. Flies from no other stock have responded in this way to copper treatment.

Upon recognition of the unexpected eye abnormalities in the copper treated *tu*^{150} stock, eight pair matings were set up with *tu*^{150} parents. Six were treated with 320 μg/ml, and two were maintained as controls in the same environment but without copper sulfate added. Following eclosion, adult flies with the eyeless phenotype were found in two of the six experimental cultures, whereas no abnormal flies were found in the control cultures.

The induced eyeless flies were inbred in cultures without copper sulfate added to the medium. Within four generations 98 ± 2 percent of the flies were eyeless. One of the new eyeless lines, symbolized tentatively *ey′*, was maintained and has now been selected and inbred for two years. Nearly 100 percent of the flies in this stock express the eyeless trait. Some expressions in the new *ey′* stock are illustrated in Figure 2. When *ey′* flies were outcrossed to wild type, Stephenville, soon after the stock was established, eight from a total of 242 F_1 progeny had slight eye abnormalities. In the F_2, 54 of the total 269, or 20 percent, had eye abnormalities, many of which were more severe than those observed in the F_1. Studies designed to investigate the genetic mechanism and the location of the gene or genes involved are in progress.

When 0.001 M copper sulfate was injected into the bodies of adult male and female flies, and the injected flies were mated, four of 501 progeny had eye abnormalities. When the injection-induced eyeless flies were mated among themselves, the trait was established and maintained in nearly all of the individuals generation after generation. The phenotype of the injection induced flies was similar to that induced in the feeding experiments. None of the 456 progeny of the controls injected with Ringer's solution showed the eyeless trait.

Relative viability of the six experimental Drosophila stocks in response to equally spaced concentration levels of iron ammonium sulfate ($Fe(NH_4)(SO_4)_2$) is illustrated graphically in Figure 3. The numerical results are summarized in Table 1. Percentages of eclosion are based on samples of 200 larvae. Analysis of variance showed a significant difference between the wild stocks (Stephenville and Canton) compared with the other four stocks tested. The tumorous head stocks were different from the melanotic tumor stocks in mortality response to the iron treatment. No difference was detected between the two wild stocks nor between the two tumorous head stocks, but a significant difference was observed

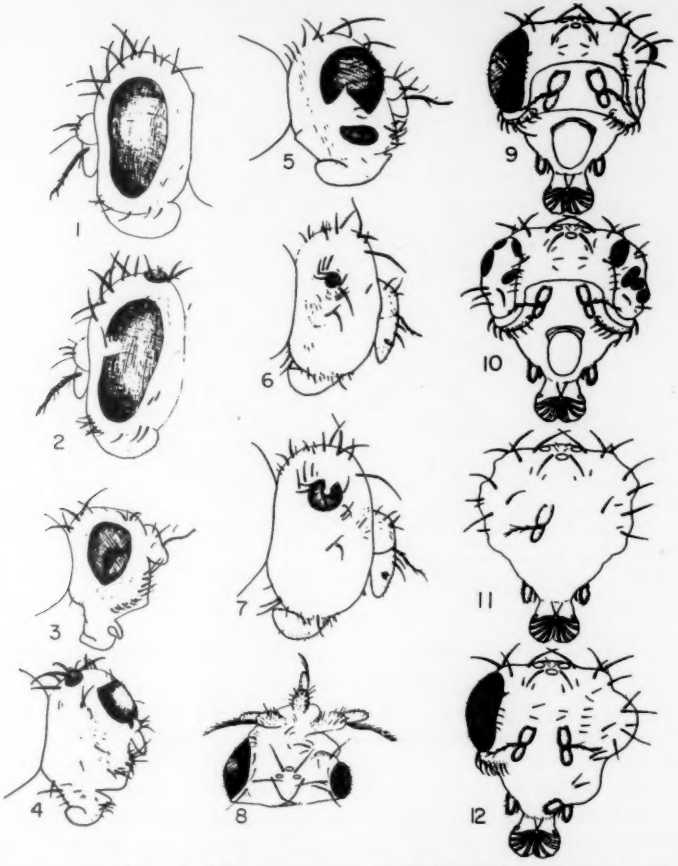

FIGURE 2.—Expressions of eyeless phenotype in inbred lines resulting from copper treatment: 1. Side view of normal head. 2. Eye indented on anterior margin by bridge of bristle-bearing tissue. 3. Moderate symmetrical decrease in eye size with inward depression in contour of eye. 4,5. Eyes with odd-shaped segments split off. 6,7,9. Small segments of eye with facets bulging out as though on a stalk. 8. Dorsal view of head showing extra antennae. 10. Eyes segmented into isolated sections. 11. Both eyes completely missing (eyeless) and one antenna missing. 12. One eye missing, one palpus duplicated.

between the two melanotic tumor strains. Variations between treatments were also significant. Total mortality of all groups increased as the iron compound was increased. The curve of response exhibited a linear trend within the range of the treatment levels.

The iron compound, like copper sulfate, prolonged the developmental period. Eclosion began in 15 days after the eggs were laid compared with 14 days for

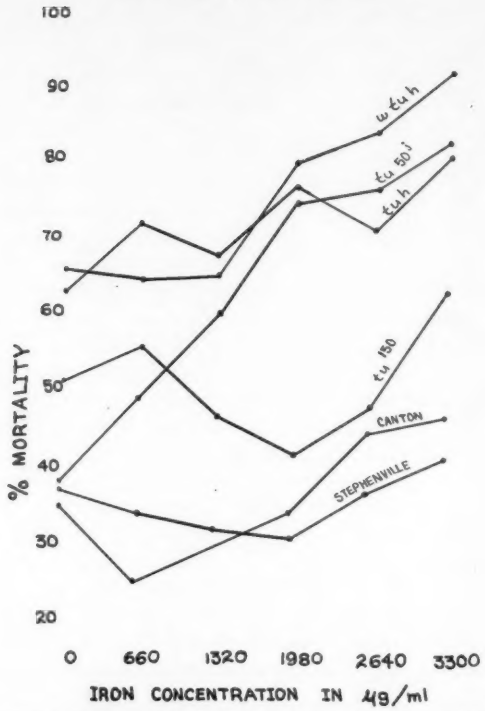

FIGURE 3.—Percentage of larvae which failed to reach adult stage when different levels of iron ammonium sulfate were added to food medium.

TABLE 1

Percentages of flies to reach adult stage grouped by treatment with iron ammonium sulfate

Stock	Treatment 3300 µg/ml	2640 µg/ml	1980 µg/ml	1320 µg/ml	660 µg/ml	Control
Canton	52	54	65	69	74	64
Stephenville	57	62	68	67	64	62
tu-h	18	28	22	31	27	39
w tu-h	7	15	19	34	36	34
tu^{150}	36	51	57	53	45	49
tu^{50j}	19	23	24	39	50	61
Treatment means	31.5	38.8	42.5	48.8	49.3	51.5

copper treatment and ten for the controls. A significant decrease in tumorous head expression and an increase in melanotic tumors were associated with the increased concentration of iron in the medium. This observation was verified in larger experiments, and analyses were made to distinguish the variation caused by the differential toxic effect on the flies and the direct effect of the treatment. A high correlation of 0.76 (r (0.05) = 0.576) was found between the decrease

of tumorous head expression and mortality. The flies expressing the tumorous head trait in the *tu-h* cultures were apparently more susceptible to the iron compound than those which were wild type in appearance.

This same question arose in a previous study (SIMMONS and GARDNER 1958) in which tryptophan was added to the medium of tumorous head flies, and the proportion of flies expressing the trait was decreased. Results of recent experiments (Table 2) have shown that the decrease is due to a differential toxicity response between flies expressing the trait and those appearing as wild type, rather than a direct effect on the genetic-physiological mechanism of the developing flies. The difference in the percentage of abnormal flies (27 percent) was not significantly different from the differential percentage (24 percent) in survival.

The culture medium in which the tryptophan experiments were conducted was examined under magnification to locate the dead organisms and to determine the developmental stage when death occurrred. The dead eggs, larvae, and pupae which could be located were classified as follows:

Developmental stage	Number located	Percent of total
Egg	48	17
First instar larva	30	11
Second instar larva	54	19
Third instar larva	106	38
Pupa	40	14

It was observed that many third instar larvae seemed unable to pupate. The larvae grew to a very large size, and dark melanotic bodies appeared throughout their abdomens.

Melanotic tumor incidence was progressively increased with increased levels of the iron compound. A correlation of 0.67 (r (0.05) = 0.576) was found between mortality and melanotic tumor incidence. Whether the wild appearing flies were more susceptible to iron treatment, or the additive influenced melanotic tumor production, has not been determined. In the previous study (SIMMONS and GARDNER 1958) 0.6 percent tryptophan added to the medium was shown to induce melanotic bodies in about eight percent of the flies in one wild stock (Stephenville). Other studies (GLASS and PLAINE 1955; KANEHISA 1955; MITTLER 1952; PLAINE and GLASS 1955) have shown that tryptophan increases the expression

TABLE 2

Comparison of the expression and survival rate of tumorous head with and without 0.5 percent L-tryptophan added

Level	No. eggs	Female		Male		Total		Percent abn.	Percent survival*
		Abn.	+	Abn.	+	Abn.	+		
Control	1,000	260	40	300	80	560	120	82	68
0.5 percent L-tryptophan	1,000	120	113	120	87	240	200	55	44
Difference	....	...	..	...	.	...	..	27	24

* Number eclosing adults/total number of eggs.

of melanotic tumors in tumor bearing stocks. Transition metals have been related to the metabolism of tryptophan (KIKKAWA, OGITA, and FUJITO 1955), a known precursor of melanotic tumor pigment. This indicates that the observed variation in incidence of melanotic tumors results from a difference in pigmentation, which makes tumors recognizable rather than an actual induction of tumors by the iron.

DISCUSSION

The most intriguing problem arising from this study is the relation between treatment with copper sulfate and the new eyeless phenotype induced in the tu^{150} stock. Three hypotheses are being considered and tested: (1) an allele for eyeless was segregating in the tu^{150} stock but was not expressed because modifiers had accumulated favoring the wild type, normal eye. Copper sulfate in the medium interacted in such a way that those flies with the genetic background most favorable to the eyeless expression showed a slight but visible abnormality. When these flies were selected and mated among themselves, modifiers favorable to the expression were accumulated with the eyeless allele, bringing about the expression independently, i.e., not requiring copper sulfate. (2) Genetic assimilation similar to that described by WADDINGTON (1957). (3) Copper sulfate influenced some controlling element and brought about a directed change at the eyeless locus.

The penetrance and expressivity of the mutant eyeless are known to decrease when eyeless flies are mass cultured in the laboratory over long periods of time (SPOFFORD 1956). Apparently modifiers acting against the abnormality are selected, and flies carrying *ey* alleles eventually become almost, if not completely, indistinguishable from wild type. No such allele is known to have been introduced into the tu^{150} stock in our laboratory, and during the period of several years while this stock has been carried in our laboratory the eyeless phenotype has never been observed in untreated flies.

WADDINGTON (1957) treated wild type Drosophila pupae with heat and obtained aberrant flies, some of which lacked posterior crossveins in their wings. Crossveinless flies were selected and heat treatment was continued generation after generation. After about 14 generations the heat treatment was discontinued, but some of the flies continued to express the crossveinless characteristic. Stocks were built up from these flies which eventually had a high percentage of crossveinless individuals. Similar experiments were conducted with ether treatment. A phenotype resembling the mutant bithorax was selected, and after 28 generations it became established, i.e., assimilated, even though the ether treatment was discontinued. The eyeless phenotype in the present study became visibly distinguishable in the first generation of copper sulfate treatment. Only four generations were required to build up a stock with nearly 100 percent eyeless individuals.

Since no other stocks have responded in this way to copper treatment, there must be a particular genetic background already established in tu^{150} or an instability which makes possible a genetic change. Copper sulfate may stimulate some controlling element to bring about a directed chemical change at the eyeless gene locus. RAPOPORT (1939) showed this locus to be relatively unstable, but there is

no precedent for a chemically directed response even in a particular stock.

Many chemical agents are now known to induce mutations, but there is no record of a particular chemical inducing a specific mutation and leaving the other genetic material unaffected. Mutations have been found to occur more or less at random, i.e., one locus is as likely to mutate as another. In the present study the eyeless phenotype first occurred following the introduction of copper sulfate into the food medium. When the chemical was injected directly into *tu*^{150} parent flies a few progeny were eyeless. In each case eyeless flies were selected, and nearly 100 percent expressed the trait within four generations of inbreeding.

Copper sulfate has been identified as a mutagenic agent (HERSKOWITZ 1951). LAW (1938) introduced this chemical into larvae of *Drosophila melanogaster* by injection and also into fertilized eggs by diffusion from the medium, and found it to induce lethal mutations at a rate greater than one percent. The induced lethals, however, were not genetically identical, i.e., they did not involve the same gene locus. KURODA and TAMURA (1956) have shown that copper ions added to a synthetic medium culturing abdominal melanotic tissue will influence tumor proliferation. KANEHISA and POULSON (1958) have described a nucleo-cytoplasmic interaction in the control of copper accumulation in *Drosophila melanogaster*. POULSON, BROWN, HILSE, and RUBINSON (1952) have studied copper metabolism in four species of Drosophila. RAPOPORT (1939) found a number of chemicals, mostly heavy metals, that produce phenocopies (nonhereditary copies of mutant phenotypes) when added to the food medium of Drosophila. The chemical agents were highly specific in the type of phenotype produced. SANG and MCDONALD (1954) were able to direct to some extent the production of specific phenocopies in Drosophila when they induced eyeless expression by treatment with sodium borate. GOLDSCHMIDT and PITERNICK (1957) directed the production of phenocopies with different boron salts. The present expression of eyeless must be associated with a permanent change in the genetic material or a recombination effect rather than a phenocopy.

SUMMARY

Copper sulfate and iron ammonium sulfate at different concentrations were added to the food medium of six *Drosophila melanogaster* stocks (*tu*^{50j}, *tu*^{150}, *tu-h*, *w tu-h*, Stephenville, and Canton). Copper sulfate treatment produced no significant change in the expression of melanotic tumors or tumorous head phenotype. Increased copper sulfate levels to 960 μg/ml were accompanied by a linear increase in mortality in both larval and pupal stages, increased duration of larval development, and differences in mortality among tested stocks. A phenotype resembling that of the mutant, eyeless, was induced in flies from the *tu*^{150} stock by copper sulfate treatment. Flies showing the new trait were selected and inbred. A new stock in which nearly 100 percent of the flies showed the new eyeless phenotype was developed and maintained. Iron ammonium sulfate treatment at levels to 3300 μg/ml increased the incidence of melanotic tumors in the *tu*^{50j} and *tu*^{150} stocks but decreased the proportion of tumorous head flies in the *tu-h* and *w tu-h*. Increased levels of the iron compound were accompanied by a linear increase in mortality, increased duration of larval development in the melanotic

tumor and tumorous head stocks, and differences in mortality among the tested stocks. The decrease in expression of tumorous head abnormalities when tryptophan was added to the food medium (SIMMONS and GARDNER 1958) was attributed to differential toxicity response rather than the genetic-physiological mechanism of the developing flies.

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THE GENETIC BASIS FOR THE CONVERSION OF MENTHONE TO MENTHOL IN JAPANESE MINT[1]

MERRITT J. MURRAY

A. M. Todd Company, Kalamazoo, Michigan

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THE major components of Mentha oils are a principal ketone, and its related alcohol and ester. Since these compounds account for 75–85 percent of the oil by weight, an elementary genetic understanding of their origin and relationship would seem important to any plant breeding work.

In this paper, we wish to consider the genetic relationship between the ketone (menthone) and its related alcohol (menthol), using *Mentha arvensis* L. var. piperascens Briq. $2n = 96$ (Japanese mint) as the low menthone-high menthol parent. For a high menthone-low menthol parent (nonmenthol by odor), we chose fertile menthone-odored S_1 strains of either *M. spicata* L. ($2n = 48$) or *M. crispa* L. ($2n = 48$). Most S_0 strains of these species have carvone and a spearmint odor. MURRAY and REITSEMA (1954) have shown that carvone (*C*) is dominant to menthone (*c*). SIEVERS, LOWMAN, and RUTTLE (1945) have previously reported that Japanese mint crossed to *M. spicata* L. gave several hybrids having menthol, and they further investigated the commercial value of these plants. Recently, hybrids of this cross have been studied by IKEDA and UDO (1957). They found menthol hybrids and also hybrids with carvone, the ketone of spearmint. While segregation was known to exist in these interspecific hybrids, a genetic analysis had not been undertaken.

EXPERIMENTAL DATA

Chemical composition and odor of parents and hybrids: The oil of the parental clone of Japanese mint has 17.6 percent menthone and 67.5 percent menthol (Table 1), and solidifies on cooling. Both the oil and the crushed herbage smell strongly of menthol and give a pronounced cooling sensation. In contrast, the oil of the high menthone-low menthol parent (S_1 strain 340 of *M. crispa*) has 56.1 percent menthone and 3.7 percent menthol. The oil or crushed herbage of this strain has a strong menthone odor which consists of a hot burning sensation in the back portions of the nasal passages. There is very little preodor in high menthone strains, and the unwary smeller (failing to detect an initial odor) may inhale sufficient oil to be very painful and to temporarily inactivate smell for several minutes. In the presence of 40–65 percent menthone, one does not detect 3–25 percent menthol from smelling the herbage. Conversely, the hot menthone

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TABLE 1

*Chemical composition by standard chemical assay**

Major chemical components in percent	Japanese mint	Hybrids			S_1 *M. crispa* strain 340†
		1	2	3	
Menthone	17.6	11.0	20.3	9.2	56.1
Menthol	67.5	57.5	67.3	65.5	3.7
Ester	5.9	24.0	7.7	14.7	2.5
Other	9.0	7.5	4.7	10.6	37.7

* Oil distilled from mature herbage, since immature herbage yields oil with relatively higher menthone and lower menthol values.
† The S_0 vegetatively propagated clone of *M. crispa* has carvone and its related alcohol and ester in similar amounts.

odor from 10–20 percent menthone is less evident in the presence of 60–75 percent menthol. One may smell pulegone, a related ketone, as a faint weak musty nail polish preodor in many *M. crispa* S_1 strains, since this ketone always occurs with menthone.

Certain F_1 hybrids of Japanese mint and *M. crispa* strains are menthone odored (like the S_1 *M. crispa* parent) and do not have detectable menthol in the herbage odor. Other hybrids (like the three whose assays are given in Table 1) have a strong menthol herbage odor and are similar to the Japanese parent in their assay values. This segregation may be studied accurately from the herbage odor of mature plants. This is a distinct advantage since it would be impossible to classify large segregating progenies if each plant had to be chemically assayed.

Inheritance data: A self-pollinated progeny of Japanese mint consisted of 101 plants with a menthol odor compared to 33 with a menthone odor. The 3:1 ratio ($P = 0.9$) suggests that Japanese mint is heterozygous for a single genic difference with the genotype *Rr*. Plants with the recessive genotype *rr* have menthone but are unable to convert much of it to menthol. We know further that this strain of Japanese mint has the genotype *cc* since it breeds true for menthone. The 3:1 ratio obtained from smelling the herbage has been verified by infrared assay of an additional small progeny of 47 selfed plants. This was a random sample since all individuals were assayed. These data presented in Table 2 indicate that all menthone-odored individuals have small amounts of *l*-menthol. From the assay data, we have a ratio of 35 high menthol-low menthone to 11 low menthol-high menthone with one individual needing reassay. This is in good agreement with our foliage odor classification of 35 menthol to 12 without apparent menthol. Infrared analysis, as performed here, has a 3–5 percent error in the midrange, and there is a tendency to greater exaggeration of the very low and very high values. While there is admittedly less precision in our measurements of the very low menthol values, all individuals have at least small amounts of menthol. The menthol values over 80 percent are likewise exaggerated.

Upon the basis of the F_2 data, we have postulated a *cc Rr* genotype for the parental clonal strain of Japanese mint. This genotype has been verified in two ways. We obtained a backcross progeny by crossing Japanese mint to selected S_1 strains of *M. crispa* with a *cc rr* genotype. These *M. crispa* strains have a strong

TABLE 2

Assays of 47 S_1 Japanese mint individuals

Classes in percent	Number of individuals in each class for:		
	Menthone	Free menthol	Ester
0–5	0	2†	14
6–10	4	6†	18
11–15	22*	3†	6*
16–20	4*	0	7
21–25	4	0	4*
26–30	1	2†	0
31–35	1	1	0
36–40	0	3	0
41–45	0	3	0
46–50	0	1	0
51–55	4†	0	0
56–60	3†	0	0
61–65	3†	4*	0
66–70	1†	7	0
71–75	0	4	0
76–80	2†	4	0
81–85	0	6	0
86–90	0	2	0
91–95	0	1	0

* Denotes position of two control unselfed Japanese parents.
† Denotes individuals without menthol by smell.

menthone odor but no menthol odor. They breed true. For example, the selfed progeny of strain 280 with a menthone odor consisted of 127 menthone-odored individuals. Strain 340 with a selfed progeny of 124 individuals bred true for menthone. Thus, we are certain that these strains have the homozygous recessive genotype *cc rr*.

The interspecific cross between Japanese mint (*cc Rr*) and *M. crispa* (*cc rr*) is readily made, and we grew 2,271 individuals in our backcross progeny. Of these, 1,157 had a menthol odor and 1,114 had a menthone odor. Since we obtained a 1:1 ratio with good probabilities ($P = 0.4$), we may consider our genetic hypothesis correct.

There is another way we may check these results. Japanese mint (*cc Rr*) was hybridized to *M. crispa* (S_0) with a spearmint odor and the genotype *Cc rr*. This cross gave a ratio of 99 carvone to 52 menthone to 55 menthol. We may consider this a good 2:1:1 ratio, since the P value is 0.8. This ratio indicates that the dominant allelomorph (*R*) cannot convert carvone (*C* −) to menthol. On the other hand, we cannot conclude that the dominant allelomorph (*R*) has no effect on a carvone plant (*CC* or *Cc*). In fact, there is evidence to the contrary. The spearmint-odored individuals from the above cross are of two types in about equal proportions. One class is like the S_0 *M. crispa* parent (*Cc rr*) with a relatively strong carvone odor and a hot after-odor sensation. The other class (presumably *Cc Rr*) has a weak carvone odor, as though the plants were low yielding.

This odor could logically be expected from individuals that had converted a high percentage of the carvone to its related alcohol. Our assumption based solely on odor has not been verified by alcohol determinations since the problem requires considerable chemical research on the origin, derivation, and identification of alcohols in a spearmint (REITSEMA 1953). We do know that both odor types have carvone and dihydrocarvone and are not correlated with the segregation of other ketones. An independently inherited ketone factor (*A*) is not considered here since it is not segregating in these crosses. The complete genotype of all homozygous menthone strains is *cc AA*.

Suggested mode of gene action: The conversion of menthone to menthol is essentially a chemical reduction, and it would seem that the dominant allelomorph may exert its action through an enzyme, a reductase. We can infer this logically from the fact that the gene seems to be specific for the reduction process without regard to the specific basal ketone compound.

DISCUSSION

From a phylogenetic point of view, *M. spicata* and other species of the genus with low alcohol values would appear to have the genotype *rr*. Their typical oil and herbage odors are those of the major ketone that normally comprise 50–65 percent of the oil.

The criticism may be made that the conversion of menthone to menthol in the plant has never been conclusively established by direct chemical means. This is apparently true, but the assumption has long been taken for granted. Oil from immature herbage has higher menthone and lower menthol values than that from mature herbage. The total of the two components menthone and menthol is a very constant value in oil samples of a given clonal species. The same total should not necessarily be expected for each of the hybrids in Table 1 since they are genetically different. While menthone is readily converted to menthol in the laboratory, the process in the plant is more specific in producing exclusively the *l*-isomer of menthol.

Japanese mint is evidently not adapted to our environmental conditions in southern Michigan, for many published assays report a much higher free menthol content than that cited in Table 1. This lack of adaptation has been noted previously by YOUNGKEN (1946), who concluded that it could not be grown profitably in this country due to our high labor costs. Our strains do not grow vigorously or yield well on either muck or upland soils. They are also highly susceptible to rust, mildew, and Verticillium wilt.

SUMMARY

In *Mentha arvensis* L. var. piperascens Briq., the conversion of the ketone (menthone) to its related alcohol (menthol) is predominantly controlled by a single gene. The dominant allelomorph (*R*) of this gene induces high *l*-menthol production (40–80 percent) in all plants having menthone (*cc*). Conversely, menthone plants with the recessive genotype (*rr*) have only 3–15 percent menthol

which is usually not detectable from foliage odor. The genotype of our strain of Japanese mint is *cc Rr*. The suggested mode of gene action is that the dominant allelomorph activates an enzyme (a reductase), which reduces a large part of the menthone to menthol. The recessive allelomorph (*r*) of the gene appears to perform the same function as the dominant allelomorph (*R*) but much less efficiently. The effects of this gene on a carvone genotype (*CC* or *Cc*) are considered, and it appears that the gene is probably specific for the reduction process rather than for the basal substance, the ketone.

From a phylogenetic viewpoint, three species of the genus Mentha have high alcohol values (and appreciable amounts of derived esters), while the other species have low alcohol values.

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THE GENETIC BASIS FOR A THIRD KETONE GROUP IN MENTHA SPICATA L.[1]

MERRITT J. MURRAY

A. M. Todd Company, Kalamazoo, Michigan

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IN *Mentha spicata* L. and the related species, *M. longifolia* (L.) Huds. and *M. rotundifolia* L., the ketone constituent of the oil is important in three ways. (1) It is the principal constituent of the oil comprising 50–75 percent of the oil by weight, (2) gives the oil and herbage its characteristic odor and (3) determines the specific alcohol and ester components that may de derived from it.

MURRAY and REITSEMA (1954) reported that *M. crispa* L. (also known as *M. spicata* L. var. crispata) had the ketones carvone and dihydrocarvone, but that its selfed progeny had a ratio of three individuals with carvone and dihydrocarvone to one with menthone and pulegone. We clearly realized that this was not a complete explanation of the segregation of *M. crispa* L. or other strains of *M. spicata* L. It was impossible at that time to publish the present genetic evidence for the third ketone group since neither ketone had ever been isolated or chemically identified. We did know that certain musty-odored segregants of *M. crispa* ($2n = 48$) were genetically equivalent to the musty-odored ketone or ketones of *M. longifolia* ($2n = 24$) (also known as *M. sylvestris* L.). This relationship was not surprising from a phylogenetic viewpoint, since on the basis of morphological appearance, European botanists had long held that *M. spicata* ($2n = 48$) was an F_1 hybrid of *M. longifolia* ($2n = 24$) and *M. rotundifolia* ($2n = 24$). This simple explanation was first disproved by RUTTLE (1931) who showed that the chromosome number alone precluded this possibility. Specifically, the F_1 hybrids between *M. longifolia* and *M. rotundifolia* would have 24 somatic chromosomes, not 48. RUTTLE further accounted for the sterility of certain *M. spicata* strains by demonstrating that these might have a few more chromosomes, or a few less chromosomes, than the balanced tetraploid number of 48. She ventured the prediction that fertile strains of *M. spicata* might be found, and they do indeed exist. Our Line 1 strain of *M. spicata* and a crisp-leaved strain known as *M. crispa* L. are completely fertile, and they have bivalent pairing and a balanced tetraploid complement of 48 chromosomes.

While it is self-evident that the ketone oil components of the tetraploid spearmints ($2n = 48$) and those of the diploid prespearmint species ($2n = 24$) should be related in some genetic manner, the answer to this question is not obvious. How is the pleasant spearmint odor derived from or related to either the very musty

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odor of *M. longifolia* or the somewhat nauseating pungent odor of *M. rotundifolia*, especially since the *M. rotundifolia* odor has not been recognized in any segregating progeny of *M. spicata*?

Chemical composition and breeding behavior of M. longifolia *(L.) Huds. and* M. rotundifolia L: The principal ketone in *M. longifolia* (*M. sylvestris*) was isolated and identified by REITSEMA and VARNIS (1956) as *l*-piperitone oxide. This pure compound has the same musty odor as the herbage of the species. While the major ketone of *M. longifolia* is piperitone oxide, REITSEMA (1956) has found it always exists with some piperitenone oxide. The predominant ketone of *M. rotundifolia* is piperitenone oxide (REITSEMA 1956), and this pure compound has the characteristic apple- or pineapple-like fragrance associated with either the herbage or oil odor of this species. *M. rotundifolia* has lesser amounts of piperitone oxide. Thus, despite their evident differences in quantitative amounts of the two chemically related ketones, both *M. longifolia* and *M. rotundifolia* have the piperitone oxide and piperitenone oxide ketones.

Mentha longifolia and *M. rotundifolia* (except for the variegated standard strain) are fertile and true-breeding for their principal ketones. Their unselfed autotetraploid strains ($2n = 48$) are perfectly fertile, and their hybrids with *M. spicata* ($2n = 48$) or *M. crispa* ($2n = 48$) are equally fertile. All species strains are clonally propagated to maintain their exact identity without inbreeding.

Preliminary data on breeding behavior of M. crispa: A self-pollinated progeny of *M. crispa* had 158 individuals possessing the musty-odored ketone to 1819 individuals not having it. Once the pure ketones (piperitone and piperitenone) were isolated from *M. longifolia* and *M. rotundifolia* and identified, it was a simple matter to demonstrate by chromatography that they were actually identical to those of the musty-odored segregants derived from selfing *M. crispa*. In this preliminary data collected in 1948, we were inexperienced and unable to accurately separate, by odor, the carvone-dihydrocarvone ketone types from the menthone-pulegone ones, as in the later data presented in Table 1. Our ratio of 1819 other ketones: 158 musty-odored (piperitone) in this preliminary data ($P = 0.2$ for 15:1) and that of 1885 other ketones: 158 musty-odored from the later data (Table 1) agree in showing that 1/16 of the total selfed progeny of *M. spicata* and *M. crispa* have the third ketone group (piperitone and piperitenone). Individuals with these ketones have the double recessive genotype *cc aa* and breed true, whereas the parental species clones of *M. crispa* and *M. spicata* are heterozygous for both genes *Cc Aa*.

Interaction of C *and* A *factors:* Table 1 shows that self-pollinated individuals of either *M. crispa* or *M. spicata* with the genotype *Cc Aa* gave an F_2 ratio of 12:3:1 for the three ketone groups. If *M. crispa* or *M. spicata* individuals with the genotype *Cc Aa* are backcrossed to a pure recessive *cc aa* (*M. longifolia* or *M. rotundifolia*), a 2:1:1 ratio would be expected and was obtained (Table 2). Spearmint-odored individuals from these backcross progenies should have the genotypes *Cc Aa* or *Cc aa*. Self-pollinated *Cc Aa* individuals should give a 12:3:1 ratio, while *Cc aa* ones should give 3 spearmint-odored:1 musty-odored piperitone. The menthone-odored individuals should have the genotype *cc Aa* and should give a selfed

TABLE 1

Interaction of C *and* A *factors*

Cross or self	Carvone and dihydrocarvone	Menthone and pulegone	Piperitone and piperitenone	Total	Ratio	P value
2n *M. crispa* selfed *Cc Aa**	330	79	30	439	12:3:1	.8
2n *M. crispa* selfed *Cc Aa*	511	122	57	690	12:3:1	.09
2n *M. spicata* (Line 1) selfed *Cc Aa*	759	202	80	1,041	12:3:1	.1
2n *M. spicata* × 2n *M. crispa*	227	64	21	312	12:3:1	.7
Total	1,497	388	158	2,043	12:3:1	.02
4n *M. crispa* selfed *CCcc AAaa*	39	2	0	41	30:5:1	
4n *M. spicata* (Line 1) selfed *CCcc AAaa*	19	6	0	25	30:5:1	
4n *M. crispa* × 4n *M. spicata*	56	4	0	60	30:5:1	
Total	114	12	0	126	30:5:1	.1
4 F_2 spearmint-odored selfed†	213	0	0	213	1:0	
3 F_2 spearmint-odored selfed *Cc AA*	220	64	0	284	3:1	.3
2 F_2 spearmint-odored selfed *Cc aa*	66	0	5‡	71	3:1	<.01
3 F_2 spearmint-odored selfed *Cc Aa*	109	21	5	135	12:3:1	.3
11 F_2 menthone-odored selfed *cc AA*	0	916	0	916	1:0	
3 F_2 menthone-odored selfed *cc Aa*	0	271	45‡	316	3:1	<.01

* Odor classification of each plant carefully rechecked.
† *CC AA* or *CC aa* or *CC Aa*.
‡ Recessives deficient in wilt test.

progeny of three menthone to one musty-odored piperitone. The musty-odored piperitone individuals (*cc aa*) should breed true and did. See lower half of Table 2 for the supporting data.

In an F_2 progeny obtained from selfing *M. crispa* (*Cc Aa*), the spearmint-odored plants should have the following genotypes: Those with *CC AA*, *CC aa*, or *CC Aa* genotypes should breed true in F_3 progenies for a spearmint odor. Individuals with a *Cc Aa* genotype should segregate 12:3:1 in an F_3 progeny. An individual with a *Cc AA* genotype should give a ratio of 3 spearmint-odored:1 menthone-odored, while an individual with a *Cc aa* genotype should give a ratio of 3 spearmint-odored:1 musty-piperitone-odored in the F_3. Likewise, self-pollinated menthone-pulegone individuals from an F_2 culture should either breed true or should give an F_3 ratio of three individuals with menthone and pulegone to one having piperitone oxide and piperitenone oxide. These expectations are verified by the F_3 data in the lower portion of Table 1.

It is of interest to note that small progenies of the unselfed autotetraploid strains with the known genotype *CCcc AAaa* did not give a double recessive individual in 126 progeny. This emphasizes the need for caution in interpreting small F_2 progenies derived from polyploid plants!

In a previous paper, MURRAY and REITSEMA (1954) showed that certain strains of *M. crispa* gave an F_2 ratio of three individuals having the carvone-dihydrocarvone ketones to one having the menthone-pulegone ketones. The above individuals that were self-pollinated had a spearmint odor and the genotype *Cc AA*. This genotype should and did give this 3:1 ratio.

Deviations in ratio expectations: The F_2 ratio 330 carvone:79 menthone:30

TABLE 2

Ketone segregation in interspecific hybrids

Cross or self	Carvone and dihydrocarvone	Menthone and pulegone	Piperitone and piperitenone	Total	Ratio	P value
2n *M. longifolia cc aa* × 2n *M. crispa Cc Aa**	58	19	19	96	2:1:1	.1
4n *M. longifolia cc aa* × 2n. *M. crispa Cc Aa*	257	188	139	584	2:1:1	<.01
Total	315	207	158	680	2:1:1	.01
2n *M. longifolia cc aa* × 2n *M. spicata Cc Aa*	61	40	27	128	2:1:1	.15
4n *M. longifolia cc aa* × 2n *M. spicata Cc Aa*	145	128	94	367	2:1:1	<.01
Total	206	168	121	495	2:1:1	<.01
4n *M. rotundifolia cc aa* × 2n *M. crispa Cc Aa*	164	78	89	331	2:1:1	.7
4n *M. rotundifolia cc aa* × 2n *M. spicata Cc Aa*	136	68	66	270	2:1:1	.98
Total	300	146	155	601	2:1:1	.89
9 selfed cultures of spearmint-odored *Cc Aa*	539	89	68	696	12:3:1	.02
4 selfed cultures of spearmint-odored *Cc Aa*†	370	119	26	515	12:3:1	.04
Total	909	208	94	1,211	12:3:1	.05
4 selfed cultures of spearmint-odored *Cc aa*	227	...	54	281	3:1	.02
4 selfed cultures of spearmint-odored *Cc aa*†	365	...	112	477	3:1	.5
Total	592	...	166	758	3:1	.05
5 selfed cultures of menthone-odored *cc Aa*	...	324	100	424	3:1	.5
15 selfed cultures of menthone-odored *cc Aa*†	...	831	240	1,071	3:1	.05
Total	..	1,155	340	1,495	3:1	.05
7 selfed cultures of piperitone-odored *cc aa*	....	...	521	521	0:1	.
13 selfed cultures of piperitone-odored *cc aa*†	...	..	729	729	0:1	.
Total	...	...	1,250	1,250	0:1	.

* Each plant carefully rechecked.
† Selfed individuals from cross 4n *M. longifolia* ×2n *M. crispa* or 2n *M. spicata*. Others from cross 4n *M. rotundifolia* ×2n *M. crispa* or 2n *M. spicata*.

piperitone ($P = 0.8$) was accurately done with the odor classification of each individual rechecked. In contrast, the total F_2 data of 1497:388:158 had a P value of 0.02 and is not a good fit for a 12:3:1 ratio. This poor fit is due to an excess of 30 piperitone-odored plants and is due to misclassification. Where classification is by odor, fatigue from smelling large numbers of plants each day leads to two types of errors in this material. Plants having a very weak or bland spearmint preodor with a hot after-odor may be confused with plants having medium menthone and a medium hot afterodor if you fail to detect the evanescent weak spearmint preodor. Plants with a high pulegone-low menthone content have a weak musty preodor, a nail polish aroma and a weak hot afterodor. These plants can be confused with those hairy plants having a weak piperitone odor. This type of fatigue is difficult to avoid or to recognize, since one may smell 20 spearmint-odored plants rapidly with no more fatigue than that induced by smelling injudiciously one menthone-odored plant. Two people working together make fewer errors.

All F_3 *M. crispa* cultures were grown for plant breeding purposes on soil heavily infested with Verticillium. The resulting F_3 odor ratios (66:5 or 271:45) have the recessive class deficient, since Verticillium wilt is generally more severe on the piperitone-odored individuals, with a consequent loss of records for this class. This observation applies particularly to inbred F_3 plants which are so severely reduced in vigor that they cannot be consistently maintained over winter under field conditions.

DISCUSSION AND CONCLUSIONS

The complete genotype of 4n *M. longifolia* and 4n *M. rotundifolia* is *cccc aaaa*, and that of 2n *M. crispa* and 2n *M. spicata* is *Ccc Aaaa*. Autotetraploid *M. crispa* thus is *CCcccccc AAaaaaaa*. It seems unnecessary and confusing to use a complete genotypic formula in the text or tables, since the ratios are primarily disomic. Dominance of the genetic factors is essentially complete, and would seem to be so even in tetraploid strains where dosage differences exist. This may be deduced from the fact that carvone and menthone do not coexist in the oil of an individual plant. Piperitone is not found in any quantity with either carvone or menthone, although a residual amount (one percent) has been reported in *M. piperita* coexisting with menthone by Reitsema (1958). Perhaps careful study may likewise demonstrate a residual amount of piperitone in carvone types.

We must emphasize the fact that the related ketones, carvone and dihydrocarvone, always coexist in the oil of any spearmint-odored plant. Individuals having the dominant *C* and dominant *A* allelomorphs are not different from those having the dominant *C* and recessive *a* allelomorphs, since both types are spearmint-odored and have carvone and dihydrocarvone. They are not necessarily alike in other respects, or even in the quantitative amounts of each ketone. Individuals with the genotypes *cc AA* or *cc Aa* have the ketones menthone and pulegone, but the quantitative proportions of the two ketones vary greatly in different plants. Likewise, piperitone and piperitenone always coexist in individuals with the genotype *cc aa*, but the quantitative proportions vary geatly in different individuals. The genetic basis for a high value of one ketone (as carvone) and a low value of the related ketone (as dihydrocarvone) has not been determined.

Our data in Tables 1 and 2 are genetic proof that the *C* and *A* factors are independently inherited and give modified dihybrid ratios. To conclude, the segregation of these two genes control the development of the six principal ketone compounds known in the genus Mentha. Except for the gene or polygenes controlling the quantitative amounts of the related ketones, these two basic genes directly or indirectly influence all the major (nonterpenic) chemical constituents of Mentha oils, thus accounting in very considerable part for the principal variability found in the oil of all the species. *Mentha citrata* Ehrh., to be considered in a separate paper, constitutes the only known exception to this statement.

SUMMARY

Vegetatively propagated clonal strains of *Mentha crispa* L. (2n = 48) and of *M. spicata* (2n = 48) with the genotype *Cc Aa* and the ketones carvone and dihydrocarvone have a spearmint odor, but their selfed progeny segregates to give a ratio of 12 individuals with carvone and dihydrocarvone to three with menthone and pulegone to one with piperitone and piperitenone. Oil from an individual of the polymorphic species *M. spicata* may have either (but only one) of the three ketone groups. Individuals with the genotypes *CC AA*, *CC Aa*, or *CC aa* are true-breeding spearmints, while others (*cc AA*) are true-breeding for the ketones menthone and pulegone. Selfed spearmint-odored individuals with the genotype *Cc AA* segregate three spearmints to one menthone-pulegone, whereas other spearmints with the genotype *Cc aa* segregate three spearmints to one piperitone-piperitenone.

The very musty odor of *M. longifolia* (L.) Huds. (2n = 24) is that of pure piperitone oxide, its principal ketone. This species has smaller amounts of the related ketone, piperitenone oxide. The apple- or pineapple-like odor of *M. rotundifolia* L. (2n = 24) is that of pure piperitenone oxide, its principal ketone, but this species also has some of the related ketone piperitone. Both basic species breed true and have the double recessive genotype *cc aa*. The genetic basis for a high value of one ketone (as piperitone) and a low value of the related ketone (as piperitenone) has not been determined.

This knowledge of ketone inheritance is important in that (1) the ketone component produces the characteristic odor of the oil and herbage in these species. (2) It is quantitatively a principal component (50–75 percent) of the oil, and (3) determines the particular alcohol and ester that may be derived from it.

The segregation of two genes control the development of the six principal ketones known in the genus Mentha. Except for the gene or polygenes controlling the quantitative amounts of the related ketones, these two basic genes directly or indirectly influence all the major and measurable nonterpenic chemical consitutents of Mentha oils, thus accounting in very considerable part for the principal variability found in the oil of all the species.

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EFFECT OF OXYGEN TENSION ON THE INDUCTION OF APPARENT X0 MALES IN DROSOPHILA

SHELDON WOLFF AND DAN L. LINDSLEY

Biology Division, Oak Ridge National Laboratory,[1] *Oak Ridge, Tennessee*

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THE production of chromosome rearrangements is the result of two separate processes: the induction of at least two chromosome breaks and the subsequent rejoining of the broken ends in new configurations. It has been possible to study these phenomena independently in *Vicia faba* root-tip mitoses by dose-fractionation experiments (WOLFF and ATWOOD 1954). It was shown that X-rays caused not only chromosome breakage but also a temporary inhibition of rejoining, the extent of which is dose dependent. This inhibition of rejoining is interpreted as damage to a rejoining system (WOLFF and LUIPPOLD 1955) and is seen as an increase in the length of time that breaks produced by one dose remain available to interact, in forming aberrations, with breaks produced by a subsequent dose.

Irradiation under conditions of anoxia produces fewer aberrations than irradiation in air (THODAY and READ 1947). This oxygen effect is observed for induced damage both to chromosomes and to the rejoining system, but the damage to the rejoining system is much more sensitive to the oxygen tension at the time of radiation than is chromosome breakage (WOLFF and ATWOOD 1954). This is best illustrated by the observation that, when the doses given in air and in nitrogen are adjusted to produce equal numbers of aberrations, the breaks produced in air remain open much longer than those produced in nitrogen. The length of time that breaks are held open (e.g., by metabolic inhibitors) does not seem to affect the probability that they will participate in the formation of a scorable aberration (WOLFF 1957). Thus, although there is a much more profound effect of oxygen during irradiation on the rejoining system than on chromosome breakage, the rejoining system eventually recovers and the same number of rearrangements result as there would have been had the rejoining system been unaffected. Therefore, the decreased number of breaks accounts entirely for the decreased yield of aberrations effected by the removal of oxygen during irradiation.

Throughout the remainder of this paper sensitivity to oxygen tension during irradiation will be referred to simply as oxygen sensitivity.

An oxygen effect has been observed for many types of induced genetic damage in Drosophila. This has been attributed either to a greater amount of restitution of breaks induced in nitrogen (BAKER and VON HALLE 1953) or to a decrease in the number of breaks induced in nitrogen (LÜNING 1954). As the result of a series of experiments using dose-fractionation procedures as had been done with the plant material, LÜNING has revised his earlier view and now postulates that comparable amounts of breakage are induced by irradiation in nitrogen and oxygen

[1] Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

but that breaks produced in nitrogen are more likely than those produced in oxygen to restitute in the original configuration, producing no scorable result (LÜNING and HANNERZ 1957; LÜNING and SÖDERSTRÖM 1957; LÜNING and HENZE 1957; LÜNING 1958). He bases his hypothesis on the observations that, when a given X-ray dose is fractionated into two exposures, one in nitrogen and one in air, separated by 15 minutes, increasing the proportion of the dose administered in air results in an increase in the observed genetic effect until a dose in air of 1000r has been achieved. Any dose in air in excess of 1000r is no more effective than an equivalent dose in nitrogen, and this is true in the total dose range from 3000 to 6000r. The observation that 1080r in air followed by 2160r in nitrogen produces the same effect as 3240r in air (1080r + 2160r) whereas 6480r (4320r + 2160r) in nitrogen is less effective than 6480r in air demonstrates that 4320r in nitrogen does not saturate the oxygen-sensitive system and thus is less effective than 1080r in air, which is a saturating dose. In other words, irradiation of the oxygen-sensitive system in nitrogen results in a dose reduction factor of at least four over irradiation in air. These results indicate that there is an oxygen-sensitive component of the total radiation-induced damage that is saturated by 1000r at 0.2 atmosphere of oxygen. LÜNING believes that this damage is to the rejoining system and that the damage sustained by the genome itself (e.g., breakage) is oxygen independent. The observed saturation has been attributed to a complete inactivation of the rejoining system.

OSTER (1957) compared the effectiveness of 2800r in producing translocations in mature spermatozoa when the radiation was delivered in nitrogen, air, or pure oxygen. If there is a combination of dose and oxygen tension, e.g., 1000r at 0.2 atmosphere, which is capable of completely saturating the oxygen-sensitive system with damage, then no increase in either dose or oxygen tension should result in any further increase in effect on this system; 2800r in air should be no less effective than 2800r in pure oxygen. OSTER's work, however, demonstrates that radiation in air yields results (6.4 percent) intermediate between those from radiation in nitrogen (2.7 percent) and radiation in oxygen (17.2 percent). Thus, it is clear that postulation of a single oxygen-sensitive system damaged to saturation by 1000r in air leads to expectations that are not fulfilled.

Because of the apparent contradiction between the expectations from LÜNING's model and OSTER's observations and because of the differences between the models postulated to account for the oxygen effect in Vicia root-tip mitoses and Drosophila sperm, we have attempted to increase the resolving power of the dose-fractionation procedure by using an oxygen atmosphere in place of air. Fractionation allows variation of the dose delivered at a particular oxygen tension while a constant total dose, and therefore constant oxygen-insensitive damage, is maintained.

We have observed, in agreement with LÜNING, that as the fraction of the dose delivered in air is increased (the remainder of the dose being delivered in nitrogen) saturation is observed. When oxygen is substituted for air in an otherwise identical procedure, however, there is no evidence of saturation and the genetic effect of increasing the proportion of the dose delivered in oxygen continues to increase until the entire dose is delivered in oxygen.

MATERIALS AND METHODS

In previous experiments (LÜNING and HANNERZ 1957; LÜNING and HENZE 1957; LÜNING 1958) the incidence of *y w sn* sons produced by a cross of irradiated $+/sc^{s}$·Y males by *y w sn* females was scored. These are progeny that have inherited neither an intact X chromosome nor a normal allele of *y* from their fathers. It has been assumed that they represent cases of loss of the irradiated X or Y chromosome and that, furthermore, this loss is the consequence of chromosome breakage. We believe that there are data in the literature that provide reasons for doubting the chromosome loss origin of the *y w sn* males and point more strongly to marker loss primarily from the sc^{s}·Y. BAKER (1955, 1957) studied marker loss from a multiply marked Y chromosome sc^{s}·Y:bw^{+} ($= K^{L}bw^{+}$·bb^{+} $K^{s}ac^{+}y^{+}$, whereas sc^{s}·$Y = y^{+}ac^{+}K^{L}$·$bb^{+}K^{s}$) and observed approximately equal numbers of cases of loss of $y^{+}bw^{+}$ and *of* y^{+} alone. These both would have been scored as *y* males in the present experiments, but only the simultaneous losses of y^{+} and bw^{+} can be explained as chromosome loss. In other crosses he observed that for 31 cases of loss of y^{+} but not bw^{+}, there were 95 cases that carried bb^{+} alone. Thus for a given incidence of y^{+} but not bw^{+} or bb^{+} losses, there are at least an equal number of $y^{+}bw^{+}$ but not bb^{+} losses. The incidence of retention of the bb^{+} marker is therefore compatible with the notion that losses of y^{+} are not accompanied by loss of bb^{+} and therefore are not indicative of loss of a chromosome. We agree with earlier views that the *y* males scored are probably the consequence of chromosome breakage.

In the experiments described in this paper, $+/sc^{s}$·Y males were irradiated but the normal X was derived from Oregon-R rather than Canton-S, which had been used by LÜNING, and the irradiated males were crossed to *y* rather than *y w sn*. The males and females were mated individually in vials and a 48-hour sperm sample was collected. LÜNING's observations were based on 24-hour sperm samples or on two successive 24-hour samples. Finally, the males that he irradiated were more homogeneous with respect to age than ours and his females laid for two successive three-day periods, whereas we collected a single six-day sample of eggs. Since our results agree with his, these differences in procedure do not seem to be important.

The irradiation was performed in a lucite chamber through which various gases (nitrogen, air, or oxygen) were passed. The flies were exposed to a flow of a given gas for 15 minutes before irradiation in that gas. Two separate sets of 12 experiments were performed. All irradiations were administered with a G. E. Maxitron tube operated at 250 kvp with 3 mm of aluminum filtration, HVL 0.43 mm of copper. The dose rate was 1000r/min. The total irradiation given was either 2000 or 4000r administered in two 2000r doses separated by 15 minutes. The half-dose (2000r) experiments were performed as controls so that we might check on any departures from additivity and find the expected values after the combined (4000r) exposure. The regular progeny of all crosses were counted with an electronic fly counter (KEIGHLEY and LEWIS 1959).

RESULTS AND DISCUSSION

The results of these experiments are shown in Table 1. The data in the table are the pooled results from the two separate sets of experiments which did not differ significantly from one another. It may be seen that repetition of LÜNING's fractionation procedure, in which he used combinations of air and nitrogen, leads to repetition of his results. The effect of the total dose administered in air was greater than that when the total dose was given in nitrogen (see Table 1, Experiments 2 and 7; 0.826% *vs.* 0.492%), whereas administration of half the total in air and half in nitrogen yielded results comparable to those obtained when the total dose was delivered in air (see Table 1, Experiments 2 and 4; 0.826% *vs.* 0.863%). However, when the difference in oxygen tension between the treatments being compared was increased by replacing air with pure oxygen in the above regime, the results from half the dose in oxygen folowed by half the dose in nitrogen were intermediate between those from the total dose administered in nitrogen or in oxygen (see Table 1, Experiments 7, 11, and 9; 0.492% *vs.* 1.333% *vs.* 2.359%). These findings are presented graphically in Figure 1. It might be noted that combined treatments yield the sum of the individual fractions, indicating a linear dose response.

Although the genetic nature of the radiation-induced effect scored in the experiments described is not thoroughly understood, some generalizations can be made about the damage incurred. There is a fraction of the total damage that is attributable to an oxygen-sensitive system, some component of which (other than radiation dose) may become limiting. We may schematically represent this system as follows:

$$A \xrightarrow{\text{X-ray}} A^* + O_2 \longrightarrow \text{Damage}$$

TABLE 1

The effect of oxygen tension and fractionation on the incidence of y *males from the cross* y *females by irradiated* +/sc^8-*Y males*

Expt.	Treatment (min)	Dose I (r)	Treatment (min)	Dose II (r)	No. y ♂/total	Percent y ♂	Percent y ♂ (corrected)*	Expected if additive
1	....	2000, Air	15, Air	....	43/8405	0.511	0.453±0.078	....
2	....	2000, Air	15, Air	2000, Air	66/7460	0.884	0.826±0.109	0.906
3	....	2000, Air	15, N_2	....	47/9530	0.493	0.435±0.072	....
4	....	2000, Air	15, N_2	2000, N_2	46/4992	0.921	0.863±0.136	0.718
5	....	.....	15, N_2	2000, N_2	30/8783	0.341	0.283±0.062	....
6	....	.....	15, O_2	2000, O_2	57/7375	0.772	0.714±0.102	....
7	15, N_2	2000, N_2	15, N_2	2000, N_2	25/4545	0.550	0.492±0.110	0.531
8	15, N_2	2000, N_2	15, N_2	.....	29/9466	0.306	0.248±0.057	....
9	15, O_2	2000, O_2	15, O_2	2000, O_2	38/1572	2.417	2.359±0.392	1.946
10	15, O_2	2000, O_2	15, O_2	....	67/6498	1.031	0.973±0.126	....
11	15, O_2	2000, O_2	15, N_2	2000, N_2	45/3236	1.391	1.333±0.207	1.130
12	15, O_2	2000, O_2	15, N_2	.....	63/6960	0.905	0.947±0.114	....
13	Control	.....	....	.....	6/10,303	0.058	0.000	....

* (Observed frequency — control frequency) $\pm \sigma$.

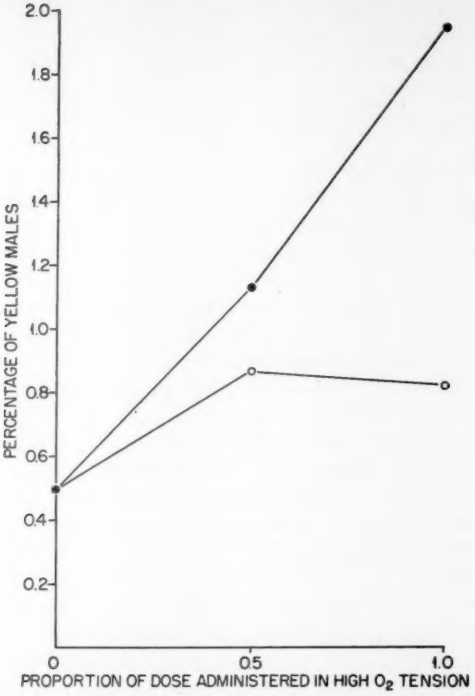


FIGURE 1.—Relation between the genetic effect and the proportion of the dose (4000r) delivered in 0.2 atmosphere (○) or 1.0 atmosphere (●) of oxygen.

where A represents a cellular component that is affected by radiation to yield A*, which in turn reacts with oxygen to yield genetic damage either directly or indirectly. LÜNING has shown when the oxygen tension is 0.2 atomsphere, no further damage to the oxygen-sensitive system can be induced by doses in excess of 1000r. He concludes that one of the cellular constituents (the rejoining system) is limiting and that once it is exhausted no further oxygen-dependent damage is possible. Our results would suggest that this may not be the case since increasing the oxygen tension to 1.0 atmosphere can increase the oxygen-dependent damage above that observed from 1000r at 0.2 atmosphere. From this observation it is possible to argue that oxygen rather than a cellular constituent is the limiting component (cf. POWERS, WEBB, and EHRET VTFJ); however, a plausible alternative is that we are observing the combined effects of several oxygen-sensitive components of damage with different properties.

For example, it is possible that the results are attributable to two independent systems. The first is sensitive to slight changes in oxygen tension (i.e., 0.0 *vs.* 0.2 atmosphere) and is saturated by relatively low doses of radiation. the second is

relatively insensitive to the difference in oxygen tension between 0.0 and 0.2 atmosphere but exhibits increased sensitivity as the oxygen tension exceeds 0.2. Such a model is similar to the one proposed to account for the effect of oxygen on Vicia root-tip chromosomes, where the relatively oxygen-sensitive system corresponds to the rejoining system and the relatively less-sensitive system to chromosome breakage.

It is not possible to describe the quantitative features of the interaction of oxygen tension and dose on radiation-induced genetic damage from the available data. Earlier experiments have varied the proportion of the total dose given at a particular oxygen tension, but have kept the tension constant. The present experiments have varied both oxygen tension and the proportion of the total dose given in oxygen, but data have been gathered from only three doses and two oxygen tensions. Many combinations of dose and oxygen tension will have to be studied before the shape of the surface, which is a function of oxygen tension, dose, and genetic effect will be known. Only then will it be possible to differentiate between the many alternative explanations of the data.

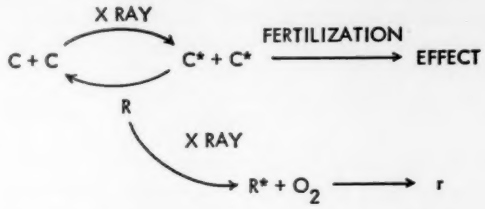
LÜNING (1958) demonstrated that there is also a component of damage that is intensity dependent. The experiment used an initial fraction of 2750r, delivered in nitrogen, in order to protect the postulated oxygen-sensitive repair mechanism, followed by a second fraction of 1650r in air after varying intervals of time. The second dose was sufficient to saturate the oxygen-sensitive system. It was observed that, when the interval between doses was 30 minutes or less, the incidence of y males produced was $\sim$30 percent greater than when the interval was 40 minutes or more. This result has been confirmed for recessive lethals (LÜNING and HENRIKSON 1959). These experiments demonstrate that there is a decay of some radiation product between 30 and 40 minutes after exposure. LÜNING believes that this product is broken chromosome ends. An intensity effect is usually interpreted as evidence that the effect measured results from the interaction of two products of radiation, at least one of which has a limited lifetime. The genetic effect measured by LÜNING shows no deviation from additivity in the present experiments. Therefore, we have an effect that increases linearly with dose and is intensity dependent; it may be represented schematically as follows:

$$A \underset{(R)}{\overset{\text{X-ray}}{\rightleftarrows}} A^* \longrightarrow \text{Damage}$$

If the damage observed is a function of the concentration of A^* produced in the cell, then it is obvious that the equilibrium established will be affected by the rate at which the radiation is administered. If, in addition, there exists a repair system (R) that is damaged by radiation, the equilibrium will be even further shifted in favor of A^*.

It is not possible from the available data to determine if this intensity-dependent system and the oxygen-sensitive system are identical.

LÜNING has amended these two generalized schemes into a more restricted picture in terms of chromosome breakage and rejoining. This may be diagrammed as follows:



where R is the rejoining system; r is the inactivated rejoining system; C is the chromosome; and C* is the broken chromosome.

Let us now examine the reasoning that has led to the hypothesis that a rejoining system plays an important role in the preceding scheme. Originally two alternatives were proposed by LÜNING and HANNERZ (1957) as possible explanations of oxygen sensitivity. They were termed differential sensitivity and rejoining. It was postulated that, were chromosome breakage oxygen sensitive (i.e., differential sensitivity), the genetic effect of 3240r administered as either 1620r in air plus 15 minutes in nitrogen plus 1620r in nitrogen or as 1620r in nitrogen plus 15 minutes in air plus 1620r in air should be intermediate between that produced by 3240r in nitrogen and 3240r in air. It was observed, however, that the fractionated treatment yields an incidence of γ males equivalent to the yield from 3240r in air (LÜNING and HANNERZ 1957; LÜNING and HENZE 1957). Similar observations have been made for sex-linked recessive lethals (LÜNING and SÖDERSTRÖM 1957) and translocations (LÜNING, personal communication). Because the postulated expectations from differential oxygen sensitivity of chromosome breakage were not realized, it was concluded that the observations favored the rejoining hypothesis. It is clear, however, that the terms differential sensitivity and rejoining both refer to oxygen sensitivity—of the chromosomes in the first instance and of the repair mechanism in the second. We believe that, although an intermediate effect of a dose-fractionation regime can be postulated as the expected consequence of the hypothesis that breakage is oxygen sensitive, it can equally well be the expected consequence of the hypothesis that the repair mechanism is the sensitive system. In the latter case, the rejoining hypothesis would have been rejected and differential breakage accepted on the basis of the same observations. In other words, we submit that the peculiar saturating effect of 1000r in air was not an *a priori* expectation for either alternative originally postulated. It should be further pointed out that had oxygen been used in place of air in the original experiments (LÜNING and HANNERZ 1957), the postulated expectation would have been fulfilled and the results would have been interpreted as favoring differential sensitivity not rejoining.

From the foregoing considerations it is clear that there is no justification for using the dose fractionation described in support of the hypothesis that there is

an X-ray-sensitive rejoining system in the mature sperm of Drosophila. Furthermore, since a rejoining system, in order to be in accord with other observations, would have to have the rather bizarre features of effecting restitution but not reunion and of operating on some broken ends but not others, it would seem more logical at this point to attribute oxygen sensitivity to some portion of the original genetic damage itself.

SUMMARY

LÜNING and co-workers have established that in Drosophila sperm there is a peculiar dose-oxygen tension interaction that results in saturation of an oxygen-sensitive system by 1000r delivered at 0.2 atmosphere of oxygen. The present experiments have demonstrated that, when the radiation is delivered at 1.0 atmosphere of oxygen, no saturation of the oxygen-sensitive system is achieved.

The results may be interpreted to indicate that at 0.2 atmosphere of oxygen and 1000r of X-rays, oxygen rather than a cellular component limits the amount of damage that may accrue.

Alternatively, the results are also consistent with the existence of two oxygen-sensitive systems, one of which is exhausted by 1000r at 0.2 atmosphere of oxygen and the other of which is insensitive to the difference between 0 and 0.2 atmosphere but is sensitive to 1.0 *vs.* 0.2 atmosphere of oxygen. This model is similar to the one proposed to account for the effect of oxygen tension on induced chromosome breakage and rejoining in *Vicia faba*, in which a rejoining system is extremely sensitive to oxygen tension, whereas breakage is less sensitive.

Therefore, there are at least two explanations of the phenomenon; however, the available data do not distinguish between them.

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POLYPLOIDY IN MORMONIELLA[1]

P. W. WHITING

Division of Biology, University of Pennsylvania, Philadelphia, Pennsylvania

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FOR the Hymenoptera in general, chromosome number is haploid for males and diploid for females, the former developing parthenogenetically, the latter from fertilized eggs. Polyploidy is exceptional.

In the ichneumonoid wasp *Habrobracon juglandis* (Ashmead), it has been shown (WHITING 1943) that some diploid males are regularly produced following inbreeding. Sex is determined by a series of multiple complementary alleles, and the diploid males are sex homozygotes. Haploids are male, and the compounds containing any two of the different sex alleles are female. If a female mates with a male having a sex allele different from her own—a three-allele cross ($xa/xb \times xc$)—all fertilized eggs are female producing. Figure 1 diagrams a two-allele cross in Habrobracon. A diploid female, xa/xb, with 20 chromosomes mates with a haploid male, xa, with ten. Only half of the fertilized eggs are female producing; the other half, the sex homozygotes, xa/xa, are male producing. However, viability of the diploid biparental males is low as compared with their sisters, and these males are near sterile. Their sperm are diploid and their few daughters triploid. There is no chromosome reduction in spermatogenesis of diploid males which appears to be similar in type to that of haploid males.

The investigations of INABA (1939) in Japan indicate some interesting racial differences between her "*Habrobracon pectinophorae* (Watanabe)" and the American or European *H. juglandis*, but the two are not specifically distinct (WHITING 1949). Cytological and genetic studies of *H. brevicornis* (Wesmael) (SPEICHER and SPEICHER 1940) indicate that this species is similar to *juglandis* in chromosomal types. In the honeybee, widely separate taxonomically from Habrobracon, sex is likewise determined by multiple complementary alleles (MACKENSEN 1955), although here sex homozygotes are always inviable. Outcrossing is the rule in natural populations of Habrobracon and the honeybee so that two-allele crosses should be very infrequent with their uneconomical production of bad eggs and diploid males.

Unlike these regular relationships between male diploidy and sex determination existing in the economy of these species, polyploidy in the chalcidoid wasp *Mormoniella vitripennis* (Walker) has arisen by mutation in laboratory cultures. In normal stock, inbreeding produces no change from the simple scheme of five-chromosome haploid males from unfertilized eggs, and ten-chromosome diploid females from fertilized eggs.

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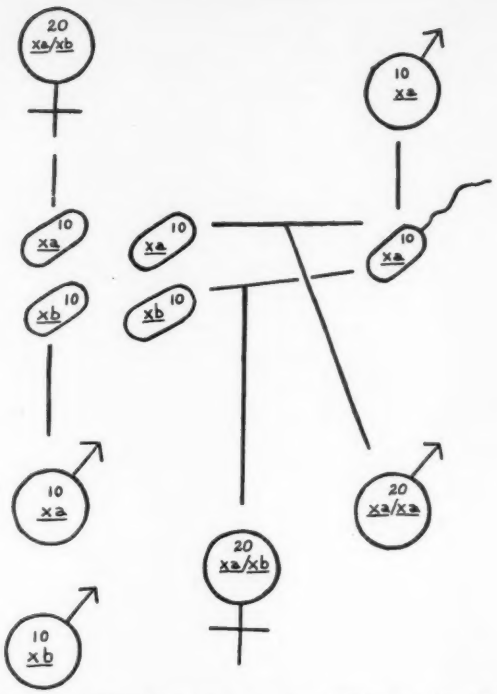

FIGURE 1.—Chromosome transmission and sex determination in a two-allele cross of Habrobracon.

Figure 2 gives the chromosome numbers as they are transmitted by crosses involving polyploidy. The pedigree shown in heavy lines indicates the method of maintaining the polypoid condition. Normal (diploid) females are crossed with diploid males of the "even" generations, F_2, F_4, F_6, etc. Because there is no chromosome reduction in spermatogenesis, all sperm are diploid and the daughters are triploid. These triploid females of the "odd" generations, F_1, F_3, F_5, etc., are separated from their haploid brothers and bred unmated. Although laying many eggs, they are of very low fecundity because most of their eggs shrivel (aneuploid). Their few progeny, "even generations", are always male, haploids and diploids (euploids). The latter are crossed with normal (diploid) females to produce the next "odd" generations of triploid females.

Light lines in Figure 2 show what happens if crosses are made other than those for maintaining the stock. If triploid females mate with haploid males, no increase in fecundity is evident. Most eggs shrivel, but a few daughters are produced from the fertilized euploid eggs. These daughters are either highly fertile normal diploids or triploids like their mothers. Triploid females have been crossed to diploid males. In addition to the few sons from unfertilized eggs and the bad eggs, there have been produced a few daughters, triploids and tetraploids as expected.

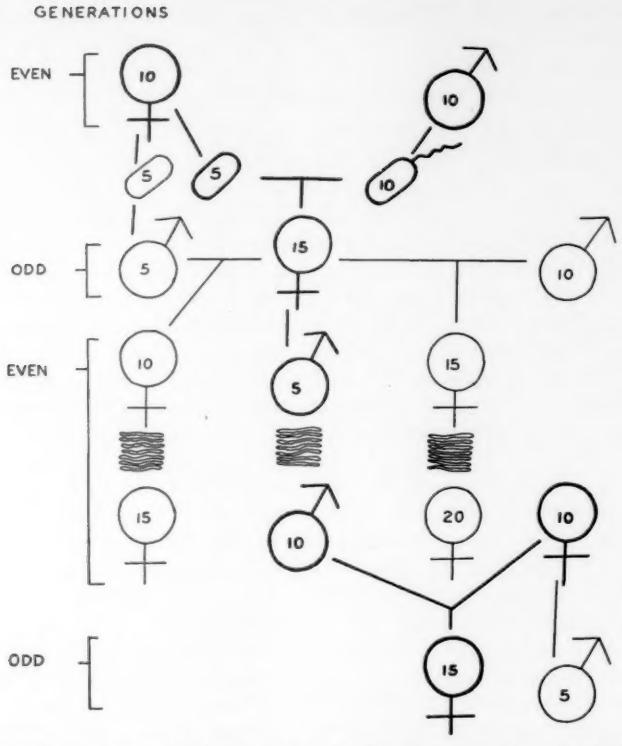

FIGURE 2.—Chromosome transmission and sex in alternated generations of polyploid stocks of Mormoniella. Heavy lines indicate method of maintaining stock.

Thus far it appears that fertilized eggs produce females, and unfertilized eggs produce males regardless of ploidy. This condition, very different from that found in Habrobracon, probably has implications for the problem of sex determination which is as yet unsolved for Mormoniella. With reference to the triploid females, both genera are alike in that they are very low in fecundity, laying many bad eggs (BOSTIAN 1936). Mormoniella diploid males are, however, unlike those of Habrobracon, very fertile siring an abundance of daughters. It is therefore possible, in this species, to carry out quantitative genetics tests with triploid females despite their low fecundity.

Diploid males tend to be larger in size and somewhat more irregular in bodily proportions than haploid. Triploid females are slightly larger than diploid but there is little, if any, greater irregularity in form. There is much size variation in Mormoniella due to amount of food available during larval growth, and this masks differences due to ploidy so that separation of diploid males from haploid and of triploid females from diploid cannot be made with certainty unless mutant trait markers are used. The few tetraploid females thus far obtained have been

identified by mutant traits. They cannot be separated otherwise from their triploid sisters.

Mutant genetics markers

The mutant genetics markers used for separating the chromosomal types are eye-color differences caused by recessive mutations in one or more of four *factors* in the "*R*" series of allelic *genes*. The word gene is here used in the sense of whatever segregates consistently as a unit in meiosis regardless of its complexity or the number of traits affected. A gene is unifactorial, bifactorial or trifactorial according to its number of factors affected by mutation. The four eye-color factors are *O*, *S*, *M* and *P*, arbitrarily placed in that order in the gene formulae. The *state* or *condition* of the factor is indicated by a symbol suggesting the eye color caused by the mutation. Thus dark red, dahlia, may be *da*.+.+.+, +.*da*.+.+, +.+.*da*.+ or +.+.+.*da* according as *O*, *S*, *M* or *P* is in a mutant condition or state. These different genes are complementary, producing dark-brown wild phenotype in the compounds, diploid males or females. Complementary genes *oy*.+.+.+ and +.*st*.+.+ cause oyster-white and scarlet eyes, being mutant in factors *O* and *S*, respectively. Mutant states or conditions of the factors form subseries of *alternatives* within the series of *allelic* genes. Compounds are wild type if they are heterozygous for recessive states in different factors, *da*.+.+.+/+.*st*.+.+; they are mutant type if they are compound for different recessive states of the same factor, *da*.+.+.+/*oy.st*.+.+, or homozygous for the same recessive state of a given factor, +.*st*.+.+/+.*st.da*.+.

Mutants in factor *O* have eye colors ranging from dark reds through bright to pale colors and finally to colorless, "oyster-white." Mutants in factor *S* have somewhat similar colors but nothing lighter than scarlet. Mutants in factors *M* and *P* are dark red, dahlia. Gene formulae are shortened to two symbols for convenience if there is no recognizable change in *M* or *P*, as apricot, *ap*.+, or vermilion, +.*vm*, for example, which are complementary. If *M* is involved in the discussion, but not *P*, the gene formula may be written with three symbols as +.+.*da* for genes *da*-845 or *da*-846, and if *P* is involved four symbols are used as +.+.+.*da* for dahlia-442.

Changes in other factors of the *R* alleles may act as recessive lethals or semilethals or male or female steriles, etc. Thus *oy*.+.*lx*, *oy.st.ly* and +.*vm.lz* are three gene formulae expressing mutant states in eye color factors *O* and *S* accompanied by lethal mutant conditions of three non-homologous factors essential to viability. The compound triploid female is wild type although somewhat reddish, probably because of incomplete dominance. Bred unmated, she produces only diploid males—wild type, scarlet and oyster—the three haploids being inviable. In the formula of such a triploid female the single maternal gene may be underlined to distinguish it from the two genes of paternal origin, as <u>*oy*.+.*lx*.+.+</u>/*oy.st*.+.*ly*.+/+.*vm*.+.+.*lz*.

Every *R*-locus gene resulting from a specific mutation is designated by a symbol in order to distinguish it from genes descended from other *R*-locus mutations.

For example, *R*-locus scarlets designated *st*-DR, *st*-474, *st*-689, *st*-808, *st*-811, *st*-814 and *st*-841 have the eye-color formula +.*st*.+.+ and differ in eye-color imperceptibly, if at all. Their most pronounced pleiotropic effects are respectively—none, egg lethality, female sterility, semilethality, pupal lethality. male sterility and near sterility with prevention of crossingover with purple body color, *pu*. normally 11 map units distant. Many other less obvious differences have been noted in other *R*-locus scarlets and the same applies to other eye colors so that it cannot be stated that any two mutations give rise to identical genes.

METHODS

Diploid males mate readily, have an abundance of (diploid) sperm and are highly fertile, siring many (triploid) females. They may, therefore, be dealt with similarly to normal haploids except that they must first be identified by eye-color markers and separated from their haploid brothers.

Because of the very low fecundity of triploid females, it is convenient to set them with relatively few host pupae. While normal diploid females, if given large host pupae in the ratio one female to one pupa, will produce many progeny exhausting the host material, a ratio of four triploid females to one pupa will produce relatively few. A much larger proportion of females is impracticable because it is necessary to furnish adequate food not only for the developing offspring but also for the freely ovipositing mothers whose many "bad" eggs (aneuploid) are as large and require as much nutriment as their few good (euploid) ones.

It is possible to increase the proportion of offspring to normal diploid mothers by furnishing more host pupae. To obtain the maximum number, transfers may be made on successive days so that fresh food is available. With triploid females living for several days and laying few good eggs, there is a loss of progeny from later laid eggs because of spoiling of food unless transfers of mothers to fresh food are made. It is noted that a few minute specimens may appear in later counts, and that small dead larvae or pupae may sometimes be found on the disintegrating host.

A few tests were made in some of the experiments reported in this paper by setting many females with a few pupae and then making frequent transfers. These transfers were made in a basin supplied with CO_2 which quickly inactivates the females so that they may be easily separated from the pupae. The wasps readily recover without injury when removed from the basin. This method is useful only if it is desired to obtain the maximum number of progeny from a limited number of mothers. It is less time consuming to use more triploid females which may be easily bred in excess.

Before the first progeny of triploid females mature, a small hole should be pricked in each puparium to facilitate emergence. Unless this is done, it frequently happens that no males will gnaw a hole, and all will perish without emergence. Males will emerge from the pricked puparium when freshly eclosed and may be quieted by CO_2 or ether to separate the diploids for breeding.

Three mutations to polyploidy

Diploid male-621.8: In stock designated *stl*-474/*oy*-DR all males are oyster, *oy*.+.+. Females are oyster, *oy*.+.+/*oy*.+.+, and wild type, +.*st l*/*oy*.+.+, in equal numbers. The latter are selected to continue the stock after they have mated with their brothers. Half the unfertilized eggs fail to hatch, and no scarlet males develop because of the lethal factor in the gene carrying scarlet.

In 1954, a wild-type male (mutant-621.8) was found in the stock. Crossed with oyster-eyed females of a stock recessive in *O* and in *S*, *oy.st*.+, he sired 72 daughters which proved to be triploid, *oy.st*.+/+.*st.l*/*oy*.+.+. Descendants were bred through February, 1955, when the strain was lost. This material, summarized, was reported (WHITING 1956a) as duplication at the *R* locus, the wild type males being +.*st.l*/*oy*.+.+, the scarlet being +.*st.l*/*oy.st*.+. These males were highly fertile like their oyster brothers, *oy*.+ and *oy.st*. In view of later findings, it is now certain that this "duplication" included the entire chromosome complex. The triploid females in the pedigree, the wild type, *oy.st*.+/+.*st.l*/*oy*.+.+, and the scarlet producing scarlet sons, *oy.st* +/+.*st.l*/*oy.st*.+, were of very low fecundity, laying many bad eggs. The scarlet females, producing oyster sons only, were diploid, +.*st.l*/*oy.st*.+, and highly fertile.

Triploid females-726.8 and 731.42: Wild phenotype compound females *vm*-MK/*oy*-DR, +.*vm*/*oy*.+, have been used in testing mutant males to determine if they are mutant in factors *O*, *S*, or both. If all daughters are wild type, the mutation involves neither of these factors, being at some locus other than *R* or in one of the *R* color factors other than *O* or *S*. If daughters are mutant type only, then both *O* and *S* have mutated, but if both wild and mutant type daughters are found, then either *O* or *S* has mutated, and it may be determined by breeding sons from these daughters which factor is affected.

During the "unstable–*O*" experiments (WHITING 1956b), mutant males were thus being crossed. Each of two sired a single exceptional wild type triploid female. Scarlet mutant male-716.14, *da.st*, sired many scarlet, +.*vm*/*da.st*, and dahlia, *oy*.+/*da.st*, daughters and the single triploid female 726.8, +.*vm*/*oy*.+/*da.st*. Tomato mutant male-714.13, *to.st*, sired many scarlet, +.*vm*/*to.st*, and tomato, *oy*.+/*to.st*, daughters, a single oyster, *oy*.+/*oy.st*, daughter and the single triploid female 731.42, *oy*.+/+.*vm*/*to.st*. The single daughter was a mutant from *to* to *oy* in the unstable *O* factor in a sperm of her mutant father. Detailed records were kept of the pedigrees from each of the mutant males including not only descendants of the triploid females involving polyploidy as evidenced by wild-type diploid males, +.*vm*/*oy*.+, but also descendants from further mutants in unstable *O*.

Although living for 20 days, triploid female 726.8 set (12/23/55) unmated, produced only 13 sons—two wild type, +.*vm*/*oy*.+, six scarlet, *da.st*, +.*vm*/*da.st*, two vermilion, +.*vm*, three oyster, *oy*.+, and one diapausing larva. One of the wild-type sons crossed with oyster females sired 11 wild-type daughters which, unmated, produced only 61 sons from 12 host pupae—13 wild type, 12 vermilion, 36 oyster and 18 diapausing larvae. The low average production of offspring from

the mutant female and her 11 granddaughters stands in contrast to the normal fecundity of the red female descendants. This indicates that these wild-type females were triploid, *oy.+/+.vm/oy.+*, and their wild-type sons diploid, *oy.+/+.vm*. Polyploidy was subsequently lost from this pedigree.

Triploid female 731.42 set (12/16/55) unmated, laid many eggs which shrivelled. Although she lived 19 days, she produced only 15 sons—three wild type, *+.vm/oy.+*, eight "red," *+.vm, to.st, +.vm/to.st*, four oyster, *oy.+*, and one diapausing larva, while her oyster, scarlet and tomato sisters proved very prolific.

Stock 850

The polyploid stock, designated stock 850, with which many experiments have been carried out, is descended from one of the wild type sons, *+.vm/oy.+*, of triploid female 731.42. He was crossed to *oy*-DR females and sired many wild-type daughters, *oy.+/+.vm/oy.+*. These produced sons of three color types—wild type, *+.vm/oy.+*, vermilion, *+.vm*, and oyster, *oy.+*, *oy,+/oy.+*. The wild-type males were selected and crossed with *oy*-250.6, *oy.st*, females. The daughters from this cross, *oy.st/+.vm/oy.+*, were reddish brown rather than wild type due to incomplete dominance. These produced males,—wild type, *+.vm/oy.+*, vermilion, *+.vm*, "scarlet", *+.vm/oy.st*, and oyster, *oy.+*, *oy.st*, *oy.+/oy.st*. The "scarlet" are diploid and are really *vm/st* compounds in factor *S*, but no haploid scarlet can be produced in later generations from them because the scarlet state of *S* is associated with oyster in the gene *oy*-250.6.

Breeding was continued in later generations by alternating crosses of normal diploid females by "even-generations" diploid males with isolation and setting of "odd-generations" triploid females. From the crosses were obtained many triploid females of low fecundity. One group of such progenies from 587 triploid females totalled 5186—only 8.835 per mother.

Males classified at this period were wild type 771, red 1883, oyster 3389. All wild type tested proved to be diploid, *+.vm/oy.+*, producing many daughters of low fecundity. Darker reds selected were all haploid, vermilion, *+.vm*, whose daughters proved highly fecund. Selected bright reds, "scarlet", were all diploid, *oy.st/+.vm*, by breeding test, and diploid oyster were also obtained by selecting the larger specimens and testing them individually.

In some cases the wild type diploid males were crossed with oyster females of mixed origin, *oy.+/oy.st*. The daughters then graded from wild type to reddish so that classification of some of them was uncertain.

Frequently the triploid females were allowed to mate with their oyster brothers —*oy.st* or *oy.+*. When mating occurred with an *oy.+* male, the wild type females produced might be diploid, *+.vm/oy.+*, from a *+.vm* egg. Such females proved highly prolific, producing vermilion and oyster sons in large numbers and no wild type males.

Purple linkage

Counts involving purple body color, *pu*, were made in some of the tests. Purple is a recessive gene taken in the wild. It shows linkage with *R* locus giving 11 per-

cent crossovers among the progeny of heterozygous diploid females. Two groups of these progenies from reddish triploid mothers may be considered. Purple was introduced in association with vermilion in the wild-type compound males in each group. The mothers were *oy.st* in both groups, but the *oy*-250.6 were nonpurple, the *oy*-NH2 were purple.

Data A: Sons from the *oy*-250.6/*vm*-MK purple/*oy*-DR, *oy.st* +/+.*vm pu*/*oy*.+ +, females are classified as nonpurple/purple into four eye-color phenotypes. There were wild type 525/6, vermilion 142/686, "scarlet" 395/30 and oyster 2216/123.

Data B: Sons of the *oy*-NH2 purple/*vm*-MK purple/*oy*-DR, *oy st pu*/ +.*vm pu*/*oy*.+ +, females are similarly classified except that the red are not separated into vermilion and "scarlet." There were wild type 88/8, red 23/225, and oyster 236/189.

Phenotypes are in general what may be expected, showing a minority of the crossover classes. The "scarlet" should all be diploid, *oy.st*/+*vm*, but a few vermilion may have been included here if they were not old enough to have attained dark color. The largest group, the oyster, are mixed for two haploid and one diploid classes—*oy.st*, *oy*.+ and *oy.st*/*oy*.+.

Chromosome assortment from a hexad

If a different *R* allele is present in each of the three homologous chromosomes of a triploid female, six genotypically different types of sons are to be expected. Relationships may be visualized as shown in Figure 3. The relative lengths of the sides of the inner, *D*, *d*1, *d*2 (diploids), and of the outer, *H*, *h*1, *h*2 (haploids), triangles express possible proportions of the six types. The parental types are maternal haploid, *H*, and paternal diploid, *D*. Recombinant haploids, *h*1 and *h*2,

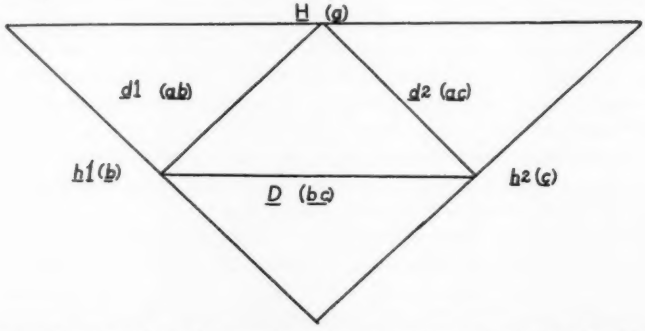

FIGURE 3.—There are shown in parentheses the six genotypes of males produced by triploid females *a*/*bc* bearing triple alleles and resulting from a cross of *aa* females by *bc* males. Possible relative frequencies of haploids, *H*, *h*1, *h*2, and of diploids, *D*, *d*1, d2 are represented by the lengths of the sides of the outer and of the inner triangles. Parental types are *H* (*a*), maternal, and *D* (*bc*), paternal. Alternative recombinant types are *h*1 (*b*) and *h*2 (*c*) of paternal origin and *d*1 (*ab*) and *d*2 (*ac*) of mixed origin.

are formed by separation of the two paternal chromosomes, and recombinant diploids, *d*1 and *d*2, are formed by union of the maternal chromosome with each of the paternal. If in every meiosis one of the chromosomes from the hexad segregates against the other two with equal frequency, it might be expected that the six types of males would be numerically equal.

Diploid males, however, tend to be less frequent than their haploid brothers. This may be due to one or more of three factors. (1) They may be innately less viable. (2) A greater proportion of their laggard larvae may die of starvation because of their slower rate of development. These two factors do not affect the theory of random chromosome assortment above stated. A third possible factor (3) is that one of the chromosomes may be included in neither daughter nucleus. It may be lost on the spindle and thus increase the number of haploids. The present study does not attempt to decide between these alternatives. Factor (2) is regarded as of minor significance. The possibility of factor (3) might be investigated cytologically.

The genetic approach can determine whether alternative recombinants are equal, *d*1 and *d*2, *h*1 and *h*2, (hypothesis of symmetry) and whether and to what extent haploids exceed diploids. It may be supposed that the ratio of haploids to diploids will be the same among the parentals as among the recombinants, $H : D = h1 \text{ (or } h2) : d1 \text{ (or } d2)$, or that the ratio of parentals to recombinants will be the same among the haploids as among the diploids, $H : h1 \text{ (or } h2) = D : d1 \text{ (or } d2)$ (hypothesis of proportionality). Figure 3 expresses the possibility that diploids are half as frequent as haploids and that in each group, a recombinant class is two thirds as great as a parental.

An ideal condition for determining the relative frequencies of the six different male genotypes resulting from segregation of triple alleles in a hexad is conceivable if each genotype could be clearly distinguished from the others. It is possible to obtain triploid females with any combination of three of the numerous *R* alleles resulting from mutation. Unfortunately, none of these make all classes of males clearly separable so that it seems necessary to reconstruct conditions from different tests.

The frequency "triangles" of Tables 1 and 2 show different sets of data (Data C–U) arranged comparably to the sides of the outer (haploid) and inner (diploid) triangles of Figure 3. A schematic arrangement is shown first, lettered as in Figure 3. Letters *H*, *h*1 and *h*2 mark the points of the inner triangle. The sides of the outer triangle might be drawn through these points and parallel to the sides of the inner triangle.

Two factor tests

In the earlier work of maintaining polyploidy and combining it with various *R* alleles, counts were made of F_2 males from various crosses. In these counts vermilion-MK, +.*vm*, was often not separated from "scarlet," +.*vm/oy.st*, the two being grouped together as "red." Similarly, peach-333.5, *pe.st*, was often not separated from "tinged," *pe.st/oy.st*, the two being grouped together as "pale."

TABLE 1

Frequency "triangles" of sons of triploid females

Groups of data triploid mothers	Phenotypes of sons	Distribution of sons in "triangles"				
		Recombinants		Parentals	Recombinants	
Schematic from Figure 3		*h*1	*d*1	*H* *D*	*d*2	*h*2
Data C						
oy. st / +.*vm*	+ 289			*oy* (611)		
	red 1061		red		*ov*	
oy. +	*oy* 1672	red		+ 289		*oy*
Data D						
oy. st / +.*vm*	+ 96			*oy* (177)		
	red 248		red		*oy*	
oy. +	*oy* 425	red		+ 96		*oy*
Data E						
oy. st / +.*vm*	+ 531			*oy* (1086)		
	vm 828		*st* 425		*oy* (425)	
oy. +	*st* 425	*vm* 828		+ 531		*oy* (828)
	oy 2339					
Data F						
pe. st / +.*vm*	+ 173			pale (428)		
	red 529		pale (145)		red (145)	
oy. +	pale 573	*oy* 384		+ 173		red (384)
	oy 384					
Data G						
+.red / +.red	+ 140			red (143)		
	red 377		+ (74)		red (74)	
oy. +	*oy* 160	*oy* 160		+ (66)		red (160)
Data H						
+.+ *.da* / +.*vm.*+	+ 200			*da* 177		
	da 177		+		+	
oy. +.+	*vm* 118	*vm* 118		+		*oy* 117
	oy 117					
Data I						
+.+ *.da* / +.red.+	+ 1205			*da* 866		
	da 866		+		+	
oy. +.+	red 818	red 818		+		*oy* 804
	oy 804					

In column 1 for each group of data, C–I, are given gene formulae of triploid females, the maternal gene above separated by a horizontal line from the two paternal genes below. In column 2 are listed the frequencies of their sons according to phenotype. These frequencies are distributed in their proper positions in the "triangles" if all those of a given phenotype are of the same genotype. Frequencies of phenotypes that include different genotypes are divided according to theoretical expectation and placed in parentheses. The positions of genotypes of listed phenotypes that cannot profitably be divided are indicated in the "triangles" by appropriate symbols.

TABLE 2

Frequency "triangles" of sons of triploid females bearing lethals

Groups of data triploid mothers	Distribution of sons in "triangles"				
	Recombinants		Parentals		Recombinants
Data J					
+. *st*. + . +			*st* 714		
oy. +.803. +		+ (224.5)		+ (224.5)	
oy. +. + .804	*oyl*–803		*oy* 300		*oyl*–804
Data K–P					
oy. +. + . +			*oy* 2879		
+. *st*. *lx*. +		+ (932.5)		+ (932.5)	
+. *st*. + . *ly*	*stlx*		*st* 1181		*stly*
Data Q					
+.*vm*.809. + . +			*vml*–809		
oy. *st*. + .805. +		*st* 17		+ 26	
oy. +. + . + .803	*oyl*–805		*oy* 31		*oyl*–803
Data R					
+. *st*.812. + . +			*stl*–812		
oy. *st*. + .805. +		*st* 238		+ 261	
oy. +. + . + .803	*oyl*–805		*oy* 290		*oyl*–803
Data S					
+.*vm*.809. + . +			*vml*–809		
oy. *st*. + .805. +		*st* 2		+ 20	
oy. +. + . + .804	*oyl*–805		*oy* 10		*oyl*–804
Data T					
+. *st*.812. + . +			*stl*–812		
oy. *st*. + .805. +		*st* 132		+ 247	
oy. +. + . + .804	*oyl*–805		*oy* 267		*oyl*–804
Data U					
oy. +. + . + .803			*oyl*–803		
oy. *st*. + .805. +		*oy* 237		+ 211	
+. *st*.812. + . +	*oyl*–805		*st* 208		*stl*–812

Arranged in general as in Table 1 but phenotypes of sons are not listed in a separate column. These are set in the "triangles" after the appropriate symbols with the wild type in Data J and in Data K–P equally divided and placed in parentheses. K–P are summarized data with the lethals 474, 809, 811, 813, 821, 824, 830 designated *st.lx* and *st.ly*. Positions of the inviable haploids on the sides of the outer "triangles" are marked by their symbols overlined and underlined.

When separation of these genotypes was attempted, the counts must be taken as approximate only because of more or less intergradation. Use of two oyster genes, *oy*.+, *oy*.*st*, results in three of the classes being indistinguishable. The theoretical numbers of these genotypes which are thus masked are calculated according to the hypotheses of symmetry and of proportionality and placed in parentheses in the "triangles" of Tables 1 and 2.

Data C: Triploid daughters, *oy*.*st*/+.*vm*/*oy*.+, of *oy*-250.6 females crossed with

wild type compound males, *vm*-MK/*oy*-DR, produced sons of the three expected phenotypes. The 289 wild type are all paternal, *D*. The 1061 red include haploid, +.*vm*, and diploid, *oy.st*/+.*vm*, (not separated) recombinants, *h*1, *d*1. According to the symmetry hypothesis, 1061 of the 1672 oyster should be recombinants. By subtraction, therefore, 611 oyster should be maternal haploids, *oy.st*, *H*. This is close to twice the paternal diploid class *D*.

Data D are Data B cited under purple linkage and resummarized in Table 1. By a method similar to that given for Data C, 177 of the oyster should be maternal haploid, *H*, which proved to be about twice the paternal diploid, *D*.

Data E of Table 1 are Data A under purple linkage. Here the 828 vermilion haploid, +.*vm*, were separated from the 425 "scarlet" diploid, *oy.st*/+.*vm*, recombinants. They prove to be about twice as frequent. By symmetry and subtraction, 1086 of the 2339 oyster should be maternal haploid, *H*, which is approximately twice the 531 paternal diploid, *D*.

Data F: Triploid daughters of *pe*-333.5 females crossed with wild-type compound males, *vm*-MK/*oy*-DR, produced the four classes of sons as shown in Table 1. The 173 wild type are all paternal diploid, *D*. The 384 oyster are haploid recombinants, *h*1. By symmetry, 384 red should be +.*vm*, *h*2, leaving 145 red, *pe.st*/+.*vm*, as *d*2. By symmetry again, 145 pale should be *pe.st*/*oy*.+, *d*1, leaving 428 pale as *pe.st*, *H*. Calculated diploids are somewhat less than half the haploids both among parentals ($173/423 = 0.409$) and among recombinants ($145/384 = 0.378$). A recombinant class is somewhat less than the parental both among the haploids ($384/428 = 0.897$) and among the diploids ($145/173 = 0.838$), but the difference is not significant.

Data G: A polyploid line was maintained for several generations in which the triploid females were *oy*-DR/*vm*-MK/*oy*-DR. Diploid males *vm*-MK/*oy*-DR of the "even" generations were crossed with stock *oy*-DR females to produce the "odd" generations triploid females. Diploid males were then bred to scarlet-DR females in order to replace the *vm*-MK gene by *st*-DR. Before this grading was completed a count of males was made in a segregating "even" generation. There were wild type 140, scarlet 352, vermilion 25, oyster 160. The scarlet and vermilion may be combined as 377 red. The 160 oyster must all be haploid, *h*1. By symmetry, 160 of the red should be *h*2, leaving 217 red to be divided between *H* and *d*2. The 140 wild type are to be divided between *D* and *d*1. By subtraction, $d1 = 140 - D$. By symmetry, $d2 = 140 - D$. By subtraction, $H = 217 - (140 - D)$. By proportionality, $D : d1$ (or $d2$) $= H : 160$. Substitute values in terms of *D*. $D : 140 - D = 217 - (140 - D) : 160$. Solve for *D*. $D = 66$. $d1$ (or $d2$) $= 74$. $H = 143$.

Diploids are slightly less than half the haploids as expected. Recombinants are insignificantly higher than parentals suggesting random assortment.

Three-factor tests

Data H: One test involving three alleles, each recessive in a different factor, was made in 1956. Wild-type diploid males, *vm*-MK/*oy*-DR, were crossed with *da*-846 females. *Da*-846 with dark-red eye color involves factor *M* alone giving

+.+.*da* as the gene formula. Seventy-three of the daughters were set in vials A, 11/26/56. The three types of haploid sons were readily distinguished. The diploid sons were all wild type as expected from the formulae. Sons, as they were counted on successive days from vials A were diploid/total—7/73 (0.096), 56/162 (0.346), 42/90 (0.467), 22/24 (0.917), totalling 127/349 (0.364) and from vials B, they were 3/69 (0.043), 31/102 (0.304), 14/47 (0.298), 25/45 (0.556) totaling 73/263 (0.278). Diploid males increase in the later counts from eggs laid by younger females as also by older females. They are, therefore, slower in development than haploids, and their frequency is not greater from eggs laid by older females.

The diploid males are approximately half as frequent as haploid males, 200/412. Their genotypes cannot be distinguished by inspection so that their distribution cannot be determined. The alternative recombinant haploids are equal, *vm* 118, *oy* 117, and each is two thirds as frequent as the maternal, *da* 177. The probability that the three classes are equal is infinitesimal.

Data I: A test involving three factors somewhat similar to Data H has recently been made. *Da*-845 females were used. This gene is similar to *da*-846, +.+.*da*, but originated from a separate mutation. The males used were wild-type compounds from the count discussed under Data G and might, then, be either *st*-DR/*oy*-DR or *vm*-MK/*oy*-DR. Each of nine of these males was set with five or six *da*-845 females resulting in a total of 1036 wild-type triploid daughters. These were separated as pupae from their haploid dahlia brothers and bred unmated. Their progenies showed that daughters of six of the males were +.+.*da*/+.*st*.+/*oy*.+.+, and that daughters of three were +.+.*da*/+.*vm*.+/*oy*.+.+. The progenies showing scarlet totalled 2,595 males—wild type 856, dahlia 607, scarlet 579, oyster 553. The progenies showing vermilion totalled 1098 males—wild type 349, dahlia 259, vermilion 239, oyster 251. Since there is no significant difference in relative frequencies between the two groups they may be summarized together as shown in Table 1, Data I. Diploid males total 1205, haploid 2485, agreeing with the preceding experiments in approximating 1:2 ratio. Among the haploid males, however, the maternal class, dahlia, is only insignificantly greater than the recombinant classes.

Counts were made from vials A after 13, 14, 15, 17 and 19 days from time of setting. These gave diploids/total sons 152/990 (0.156), 325/872 (0.373), 371/872 (0.425), 195/511 (0.382) and 73/168 (0.435), respectively totalling 1116/3413 (0.327). Vials B likewise showed an increase in relative frequency of diploid males in later counts. Counts were made after 12, 13, 14 and 20 days from time of setting giving diploids/total sons 5/51 (0.098), 33/112 (0.295), 30/69 (0.435), and 24/48 (0.500) respectively totalling 92/280 (0.329). As in Data H, the diploid males are relatively more frequent in later counts from the same vials indicating a slower rate of development. They are not more frequent among the sons of older females.

Use of lethals

Many of the alleles in the *R* series carry recessive lethals which prevent the haploid males from developing. The symbols for such genes may include an ab-

breviation for the eye color, the letter l and the mutation number as *oyl*-803. This lethal has incidence in the larval stage so that cultures from unmated heterozygous diploid females show large numbers of inviable half-grown larvae if puparia are opened after the viable class has emerged. The lethal factors may, for convenience, be expressed in the gene formulae by using the number of the mutant gene. Thus, the gene formula for *oyl*-803 is *oy*.+.803. Compounded with *st*-DR it gives wild type females, *oy*.+.803/+.*st*.+, which unmated produce scarlet sons and these inviable larvae. *Oyl*-804 has an egg lethal, non-homologous with *oyl*-803. Its gene formula is *oy*.+.+.804. Oyster-eyed diploid males, compound for the two genes are therefore viable, *oy*.+.803.+/*oy*.+.+.804. To obtain these, *pe*-333.5, *pe.st*.+.+, was used. Wild type compound females, *oyl*-803/*st*-DR, *oy*.+.803.+/+.*st*.+.+, were crossed with "tinged" compound diploid males, *oyl*-804/*pe*-333.5, *oy*.+.+.804/*pe.st*.+.+. Sons produced were scarlet viable and the inviable larvae, *oyl*-803. Daughters were wild type +.*st*.+.+/*oy*.+.+.804/*pe.st*.+.+, and "tinged" *oy*.+.803.+/*oy*.+.+.804/*pe.st*.+.+. The latter bred unmated produced the inviable *oyl*-803 larvae, the viable peach and "tinged" males and the desired viable oyster males.

Data J (Table 2): Two of these oyster males set with *st*-DR females sired 119 wild type daughters, +.*st*.+.+./*oy*.+.803.+/*oy*.+.+.804. These, bred unmated, produced sons—wild type, *st*-DR/*oyl*-803, *st*-DR/*oyl*-804, scarlet, *st*-DR, and oyster *oyl*-803/*oyl*-804. These oyster males were again crossed with *st*-DR females to produce mothers for the next generation, and this was repeated so that there were five "odd" generations of wild-type triploid females totalling 1136. These unmated produced 1463 males—wild type 449, scarlet 714, oyster 300, and an uncounted number of *oyl*-803 larvae.

The frequencies are distributed in the "triangle" under Data J, Table 2. The 300 oyster are diploid paternal, *D*, which are less than half the 714 haploid maternal, *H* (ratio 0.420) as expected. The 449 wild type should be of two genotypes, *st*-DR/*oyl*-803 and *st*-DR/*oyl*-804, which by symmetry should each be 224.5. Ratio of such a calculated recombinant diploid class to the paternal diploid is (224 5/300 = 0.748) about ¾. This is highly significantly different from equality but insignificantly different from the 2:3 ratio found for haploids under Data H, Table 1.

Tests were made which are comparable to those of Data J but which had eye color reversed respecting the lethals. *Stl*-474 carries a recessive egg lethal. This gene originated from the only *R*-locus spontaneous mutation bearing a lethal which has yet been found. Its formula is +.*st*.474. *Stl*-811, +.*st*.811, carries a recessive pupal lethal. Inviable pupae with brilliant scarlet eyes are to be found in abundance in puparia after infection by unmated compound females. None of these scarlets matures. To obtain scarlet males compound for the two genes, wild type compound females, +.*st*.+.811/*oy*.+.+.+, were crossed with wild type diploid males, +.*st*.474.+/*oy*.+.+.+. Daughters of the two expected genotypes are indistinguishable, both wild phenotype. When they were bred unmated, there were produced males of the three expected eye colors—wild type, scarlet, oyster.

Data K: These viable scarlet compound males were crossed with *oy*-DR females

and sired wild type triploid females, *oy*.+.+.+/+.*st*.474.+/+.*st*.+.811, which produced sons of the three expected eye colors. Generations were alternated by crossing the compound scarlet males to *oy*-DR females and breeding the wild type triploid females unmated to produce seven "even" generations of segregating males. From 1174 such triploid females there were obtained 1744 viable sons —wild type 483, scarlet 393, oyster 868, and many uncounted inviable scarlet pupae, +.*st*.811. The 393 scarlet are diploid paternal, *D*, which is less than half the 868 haploid maternal, *H*, (ratio 0.441). The 483 wild type should be of two genotypes, *oy*-DR/*stl*-474 and *oy*-DR/*stl*-811, which by symmetry should each be 241.5. Ratio of a calculated recombinant diploid class to the paternal diploid is 241.5/393 = 0.615 which is close to the 2:3 ratio for haploids found under Data H, Table 1.

Vml-809, +*vm*.809, carries a "lethal" with incidence at time of eclosion. Some of the males are unable to eclose; others complete eclosion but are unable to walk about, feed or mate. The impairment, therefore, stands midway between a pupal lethal and a male sterile. Eye color is an intense dark red with considerable black pigment. In compound with scarlet or with a pale eye color such as *pe*-333.5, *pe.st*, tinged-277, *ti.st*, or an oyster scarlet, *oy.st*, the females have brilliant red eyes closely resembling scarlet, and this applies also to the compound diploid males.

Data L: Tests were made combining *vml*-809 with the pupal lethal *stl*-811. Wild type compound females *vml*-809/*oy*-DR were crossed with wild type compound diploid males *stl*-811/*oy*-DR and the wild type triploid daughters set unmated. Viable scarlet compound males, +.*vm*.809.+/+.*st*.+.811, were thus obtained and mated with *oy-DR* females. Triploid wild type daughters *oy*.+.+.+/+.*vm*.809.+/+.*st*.+.811, were obtained in two "odd" generations. Their sons from 275 mothers totalled 287, wild type 78, scarlet 54, oyster 155 besides numerous inviables, some with bright scarlet eyes and some with intense dark-red vermilion eyes.

Data M: Tests were also made combining *vml*-809 with the pupal lethal *stl*-813 and carried out as with *stl*-811 through three "even" generations. The wild-type triploid females, *oy*.+.+.+/+.*vm*.809.+/+.*st*.+.813, produced sons, wild type 396, scarlet 228, oyster 478 and numerous uncounted vermilion inviable and scarlet inviable.

Sons of females *oy*-DR/*vml*-809/*stl*-811 and of *oy*-DR/*vml*-809/*stl*-813 total wild type 474, scarlet 282, and oyster 633. The diploid scarlet, *D*, are close to half the haploid oyster, *H*, (ratio 282/633 = 0.445). Half of the wild type, a calculated recombinant diploid class, *d*1 or *d*2, is less than the paternal diploid, *D*, (ratio 237/282 = 0.840). This deviates significantly from the 2:3 ratio found in the haploids of Data H.

Stl-830, a pupal lethal, +.*st*.830, was tested with *stl*-811, a pupal lethal, +.*st*.811, with *stl*-821, an egg lethal, +.*st*.821, and with *stl*-824, an egg lethal, +.*st*.824. In each case wild type females, *stl*-830/*oy*-DR, were crossed with wild type diploid males, compounds of the scarlet gene with oyster. The wild-type triploid daughters were set to produce scarlet compound sons which were crossed with *oy*-DR females.

Data N: In the test involving *stl*-811, 153 wild-type triploid females, *oy*.+.+.+/+.*st*.811.+/+.*st*.+.830, from three "odd" generations produced 130 sons; wild type 30, scarlet 16, oyster 84 and inviable scarlet pupae.

Data O: In the test involving *stl*-821, 824 wild-type triploid females, *oy*.+.+.+/+.*st* 821.+/+.*st*.+.830, from three "odd" generations produced 1141 sons; wild type 365, scarlet 217, oyster 559 and inviable scarlet pupae.

Data P: In the test involving *stl*-824, 1073 wild-type triploid females, *oy*.+.+.+/+.*st*.824.+/+.*st*.+.830, from two "odd" generations produced 1521 sons; wild type 513, scarlet 273, oyster 735 and inviable scarlet pupae.

Since the ratios of the three eye-color phenotypes of the viable males do not differ significantly in the three experiments with *stl*-830, the frequencies may be summarized giving wild type 908, scarlet 506, oyster 1378 among 2792 total. The diploid scarlet, *D*, are somewhat less than half the haploid oyster, *H*. Half of the wild type, *d*1 or *d*2, is less than the paternal diploid, *D* (ratio 454/506 = 0.897) differing significantly from the 2:3 ratio found in the haploids of Data H.

A summary of Data K to P is given in Table 2. The 1181 scarlet are all paternal diploid which is less than half the maternal haploid oyster (ratio 1181/2879 = 0.410) as expected. The 1865 wild type are compounds of *oy*-DR with the scarlet lethals. For each of the tests the wild type should be equally divided as *d*1 and *d*2. For the total, then, there should be 932.5 *d*1 and the same for *d*2. The recombinant classes are, therefore, less frequent than the paternal (ratio 932.5/1181 = 0.790). The chance for random assortment, that the paternal diploid class, *D*, is equal to half the sum of the recombinant diploids, *d*1 and *d*2, is infinitesimal.

Trilethal tests

By selecting alleles, each one of which carries a lethal factor, it is possible to obtain triploid females which produce diploid sons only, all three haploid types being inviable. The alleles may be so chosen that each of the diploid male types will possess a different eye color. It should thus be possible to determine whether the alternative recombination diploids, *d*1, and *d*2, are numerically equal as well as to observe a possible excess of the paternal type *D*. A lethal-bearing gene that is double recessive for color factors is available, *oyl*-805, *oy*.*st*.805. If the other two genes are an oyster lethal and a scarlet lethal, the three eye color types will be wild type, scarlet and oyster.

Oyl-805 was first combined with *oyl*-803, *oy*.+.803, and with *oyl*-804, *oy*.+.804, to obtain viable diploid compound oyster males. This was done by crossing dahlia females, *oyl*-805/*da*-GF, *oy*.*st*.805/*da*.+.+, with tinged diploid males, either *oyl*-805/*pe*-333.5, *oy*.+.803/*pe*.*st*.+, or *oyl*-804/*pe*-333.5, *oy*.+.804/*pe*.*st*.+, or with wild-type diploid males *oyl*-804/*st*-DR, *oy*.+.804/+.*st*.+. When the tinged males were used, it was possible to select for breeding the tinged triploid daughters bearing both oyster lethals and to discard the red sisters. If the wild type males (*oyl*-804/*st*-DR) were used, the two classes of daughters could not be separated; in either case any viable oyster appearing in F_2 would be the desired compound males.

Such males were obtained and crossed with compound females carrying *vml*-809 or *stl*-812. These compound females might be scarlet, +.*vm*.809/*pe.st*.+, +.*st*.812/*pe.st*.+ or wild type, +.*vm*.809/*oy*.+.+, +.*st*.812/*oy*.+.+. In either case the daughters would be of two types—tinged to be discarded and wild type bearing the three lethals to be retained.

Four groups of such "trilethal" females were obtained in each of which the scarlet-bearing gene was maternal; the two oyster, paternal. Formulae of the triploid females and frequencies of their viable sons of the three eye-color phenotypes as also the formulae of the three haploid inviables are shown in Table 2. Data Q–T. The presence of the inviable larvae *oyl*-803 and the inviable weak eclosing adults *vml*-809 were noted where expected. In group T no inviables were found, the three lethals having incidence in the egg stage.

In maintaining the trilethal stock, the oyster males obtained may be crossed with females compound for a scarlet lethal gene as already explained.

Data U: It is also possible to cross the scarlet males with females compound for an oyster lethal and to obtain trilethal females in which the same chromosomes are differently derived. This was done by crossing scarlet males, *stl*-812/*oyl*-805, with wild type females, *oyl*-803/*st*-DR. The triploid scarlet daughters were discarded and the wild type containing the three lethal factors were set unmated, Table 2, Data U. The characteristic half-grown inviable larvae *oyl*-803 were noted.

Except for Data T the frequencies of the three types of viable sons do not differ from equality, the expectation from random assortment. In T there is a highly significant reduction in scarlet below the alternative wild-type recombinants. In Q, R and S, scarlet is somewhat below wild type but insignificantly so. If the scarlet in T be increased by a factor adequate to make the scarlet in Q, R and S equal to their alternative recombinants, it becomes 157.7 which is still very significantly below its alternative group. The frequencies in T are summarized from three different groups of mothers in each of which the ratios are similar. It may, therefore, be concluded that there must be some factor in this combination that is absent in the others. Data from the trilethal females give no evidence for non random assortment of the three chromosomes from the hexad.

Fecundity of triploid females

No experiments have been carried out primarily with the purpose of determining exactly the fecundity of triploid females. Their reduction in number of offspring is so great that this, together with the shrivelled remnants of their eggs, serves to identify them readily. Experiments reported in this paper were conducted under varying conditions and no close or consistent checks were made as to number of immature inviables or of diapausing larvae present. In general, the latter were not numerous because temperature was kept uniformly high, 27°–30°C. Host pupae were, for the most part, good, but there was some variation in numbers supplied and in transfers to fresh food.

From the tests made in which the triploid mothers were counted, offspring per female were (9488/1696) 5.594 when there were no lethals present, (7219/

5323) 1.362 when two of the alleles carried lethals and (2091/1625) 1.290 from the trilethal experiment.

DISCUSSION

Randomness in segregation of genes is a fundamental principal of Mendelism, but this is limited in various ways as by linkage within a chromosome. Non random or preferential segregation of chromosomes at meiosis has been found in several species of animals and plants. Perhaps the greatest hierarchy of "preferred" chromosomes was shown by STURTEVANT (1936) in triplo-IV females of *Drosophila melanogaster*. Twenty-six different kinds of IV chromosomes named for marker gene differences were used as well as a translocation having a IV centromere and distal end with the left end of X included. These may be arranged in a series such that each will pass to the haplo-IV pole more often than any other above it in the seriation. In other words, if chromosomes A and B are tested against C, then C will "prefer" to go to the pole with that one of the other two that lies higher in the series. Because crossing over is negligible in chromosome IV these preferences involve whole chromosomes rather than individual loci and are due to something in the nature of the chromosomes themselves rather than to their combinations in the parental flies.

The breeding data with Mormoniella, reported in the present paper, indicate that among the sons of triploid females, diploids are somewhat less than half as numerous as haploids. This may be due to a viability difference or to some cytogenetic factor, possibly a failure of one of the three chromosomes from an oocyte hexad to go to either pole. Diploid males can be formed only when all hexads segregate two against one and when all the twos go to the inner pole. If one or more hexads should segregate one against one, the third chromosome being lost, only haploids or aneuploids could result. For any one of the five hexads, one against one segregation need not be frequent to account for 50 percent reduction in diploid males. This should be investigated cytologically.

The three-factor tests, Data H and I (Table 1), suggest that among the haploid sons of triploid females, the maternal type, *H*, is more frequent than either of the types of paternal origin, recombinants *h*1 and *h*2, but the ratio of recombinants is much higher in Data I than in H. Among the diploid sons from triploid females bearing two lethals, Data J–P (Table 2), the paternal type, a parental D, with two lethals is more frequent than half the sum of the two recombinant types, *d*1 and *d*2, which carry a single lethal. The trilethal tests, Data Q–U (Table 2), are inadequate to show that the parental diploid type *D* is more frequent than half the sum of the recombinant diploids, *d*1 and *d*2. It is likely that viability differences may enter here to mask the genotypic ratios.

In two-factor tests, Data C–G (Table 1), involving mutant differences in factors *O* and *S* without lethals, it is possible to extrapolate genotypic frequencies that are masked under a reduced number of phenotypes. This is done by assuming symmetry, that the two haploid recombinant classes, *h*1 and *h*2, should be equal as also the two diploid recombinant classes, *d*1 and *d*2, and by assuming proportionality, that ratio of recombinants among diploids and among haploids

should be the same. Theoretical frequencies so derived agree in making diploids much less frequent than haploids. Recombinants are here less frequent than parentals, Data E and F, or equal to parentals, Data G.

Meiosis in triploid females of Mormoniella and Drosophila may be compared. In the wasp, each hexad consists of one maternal and two parental chromosomes, but this is reversed in the fly. In Drosophila, analysis of hexad segregation is complicated by the fact that fertilization is necessary for egg development and that there are two types of sperm. Aneuploids for chromosomes II and III are inviable, and diplo-II-III eggs give rise to intersexes and triploid females of somewhat lowered viability and to rarely viable supersexes. Segregation of chromosomes from the different hexads is not at random. If one II and one III remain in the egg, the chance that one X will do so has been estimated as 1:6.5 rather than 1:1 (MORGAN, BRIDGES and STURTEVANT 1925). Only the diploid offspring from haplo-X-II-III eggs are reliable for quantitative study of crossing over or of segregation of chromosomes from a single hexad.

Studies have been made on crossing over in triploid Drosophila, and these may yield evidence regarding segregation of chromosomes from a single hexad. REDFIELD (1930), scoring the diploid offspring of triploid females, studied crossing over between five "loci" in chromosome III. To obtain "triple alleles" in order to mark each "locus" in the three chromosomes, she made use of two mutant genes so closely linked that crossovers could be ignored. She summarized the data of her Table I with respect to each "locus" separately, including parentals, and crossovers between the "loci"; she has supplied me with the data thus summarized. Each "locus" has one wild type "allele" and two mutant type. The frequencies of the traits may be given consecutively for the five "loci" on each of the three chromosomes. For one of the maternal chromosomes these were + 359, *D* 369, *ma* 361, *Sb* 369, and + 364, and for the other maternal chromosome *se* 316, *M-h* 314, + 335, + 332 and *H* 328. For the paternal chromosome they were *h* 349, + 341, *cu* 328, *bx* 323 and e^s 332. The observed excess representations for loci of the *D ma Sb* chromosome (which are statistically significant) REDFIELD attributes to a simple viability effect (personal communication). These total 1822 and the representations for the other maternal chromosome total 1625. These are in haploid recombinant eggs, *h*1 and *h*2. The haploid parental representations, *H*, paternal in Drosophila rather than maternal as in Mormoniella, total 1673.

There is, then, no evidence for preferential segregation in hexad III of the paternal contribution as against either of the recombinant maternal chromosomes. The present study shows that the parental contribution to a hexad in triploid Mormoniella females may exceed recombination classes among the progeny.

Preferential segregation of chromosomes at meiosis depending upon their combination in the parents has been shown in "monogenic" species—species with unisexual broods. Among these the best evidence is in Sciara (METZ 1938; CROUSE 1943), for this combines cytological identification with genetics markers. Oogenesis is orthodox, but in spermatogenesis the parental chromosomes are lost. Thus only the maternal chromosomes are transmitted and these become the paternal which are lost in the next generation. Preferential segregation is here de-

pendent on parental combination rather than on the nature of the chromosomes themselves as in STURTEVANT's triplo-IV Drosophila.

Preferential segregation with loss of presumably paternal heterochromatic chromosomes occurs in coccid spermatogenesis of the lecanoïd type (HUGHES-SCHRADER 1948). The differences in chromosome behavior are "not determined by the individual chromosome since heteropycnosis in a given male involves chromosomes which were euchromatic in the preceding generation."

The production of polyploid individuals, diploid males and triploid females, is a constant possibility in Habrobracon, involved in the system of sex determination. The economic wastage resulting is minimized by the fact that outcrossing is the rule, the males having long wings so that two-allele crosses tend to be infrequent under natural breeding conditions. Furthermore, diploid males are low in viability, and their near sterility insures that their progeny, triploid females, are produced very rarely.

In Mormoniella polyploidy poses no such threat, and the probability of its occurrence is, like a genic mutation, very low. The chance for it to be perpetuated in nature without artificial selection is slight for it must be transmitted through the weakly fecund triploid females. Consequently, there seems to have been no need for selection to reduce the viability or the fertility of diploid males as in Habrobracon. The short wings of the Mormoniella males are a factor giving a high probability of inbreeding. A sex-determining mechanism of the Habrobracon type would, therefore, be economically disadvantageous.

The problem of sex determination in the seven invertebrate groups widely disparate taxonomically but similar in showing male haploidy was "solved" (WHITING 1945) by extending to them the complementary allele theory. That *multiple* complementary alleles are not necessarily involved was later shown for the chalcidoid wasp Melittobia (SCHMIEDER and WHITING 1947). In Melittobia the males are very handicapped, flightless and sightless, and consequently there is much inbreeding. Female ratio is very high, over 95 percent, and there are no diploid males. Unmated females, or mated females that have exhausted their sperm supply, lay very few eggs. The food supply, the host larva, must not be wasted. Sex determination of the Habrobracon type would here be very uneconomical.

In the honeybee it has been shown (MACKENSEN 1955) that a series of multiple sex alleles exists as in Habrobracon. There are no diploid males produced by artificial inbreeding, but half the fertilized eggs from two-allele crosses fail to hatch. Outcrossing is the rule in nature, and complete inviability of sex homozygotes indicates a highly efficient reproductive economy exceeding that of Habrobracon.

The chalcidoid wasp, Pteromalus, according to the genetics data of DOZORCEVA (1936a) reviewed by WHITING (1940) may follow the Habrobracon pattern, but her tests were incomplete, and no diploid males were found. Her cytological work pictured W and Z chromosomes (1936b), but B. R. SPEICHER (personal communication) was unable to find such in American stock. The males of Pteromalus have long wings and fly readily, resulting in outcrossing so that multiple sex alleles would not be a handicap.

The five genera of Hymenoptera mentioned in this discussion may be arranged in a series beginning with (1) the honeybee, Apis, of the superfamily Apoidea with multiple complementary sex alleles, much outcrossing, and two-allele crosses *very* infrequent. (2) Habrobracon of the Ichneumonoidea has similar sex determination and outcrossing the rule but chance of two-allele crosses greater. (3) Pteromalus and (4) Mormoniella of the superfamily Chalcidoidea, tribe Pteromalini, are very close taxonomically. Nevertheless, they differ in that Pteromalus has long-winged males; hence there is less chance for inbreeding and two-allele crosses. The Habrobracon system of multiple sex alleles may apply. (5) Melittobia of the Chalcidoidea, placed in the Eulophidae, a different family from the last two, has its economics adapted for considerable inbreeding and has a sex-determining system differing, as in Mormoniella, from that of Habrobracon and Apis.

It appears that the system of sex determination is determined not by taxonomic affinity but by amount of inbreeding occurring in nature.

What then is this system that is alternative to the system of Habrobracon? There is as yet no evidence against a complementary system or one with digametic females. The difference is between supplementary hypotheses, multiple sex alleles *versus* multiple factors, selective syngamy or differential maturation. The last, shown in the present paper for triple alleles in triploid females, may later be found to govern the sex factors.

VANDEL (1939) has pointed out that for animals of the Abraxas type, with W Z females, sex ratio may deviate widely from equality and that this deviation may be caused by genetics or by environmental conditions affecting the distribution of the chromosomes at meiosis. Bearing this in mind, a search for a sex-linked trait or an unequal chromosome pair may prove more fruitful than further speculation.

SUMMARY

Three separate spontaneous mutations to polyploidy, diploid males, triploid females, have been found in Mormoniella. Diploid males are highly fertile producing diploid sperm only. When crossed with normal diploid females, they sire many daughters, all triploid. Triploid females lay many eggs, but very few hatch (euploid). Unmated triploid females produce males only, diploid and haploid, the former less than half as frequent as the latter. Triploid females mated to haploid males produce triploid and normal diploid daughters. Mated to diploid males they produce triploid and tetraploid daughters. Fertilized eggs produce females; unfertilized eggs produce males regardless of ploidy.

Genetics markers are mutant eye colors due to multiple complementary alleles. A triploid female carrying three alleles produces sons of six genotypes—three haploid, three diploid. Lethal factors in two or three of the alleles kill haploid males making it possible to identify the remaining classes and to determine the ratios of the diploid types.

Parental classes of sons, maternal haploids and paternal diploids, tend to be more numerous than the recombinant haploids and diploids, respectively. Recombinant haploids are formed by separation of the two paternal genes, and recombi-

nant diploids are formed by union of each of these with the maternal gene in meiotic segregation.

This preferential segregation favoring parental types is compared with retention of maternal chromosomes only, in spermatogenesis of Sciara and lecanoïd coccids. It is suggested that preferential segregation of W *versus* Z chromosomes in normal diploid oogenesis may maintain reproductive economy in sex determination for many species with haploid males.

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